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The Journal of The American Society of Hematology

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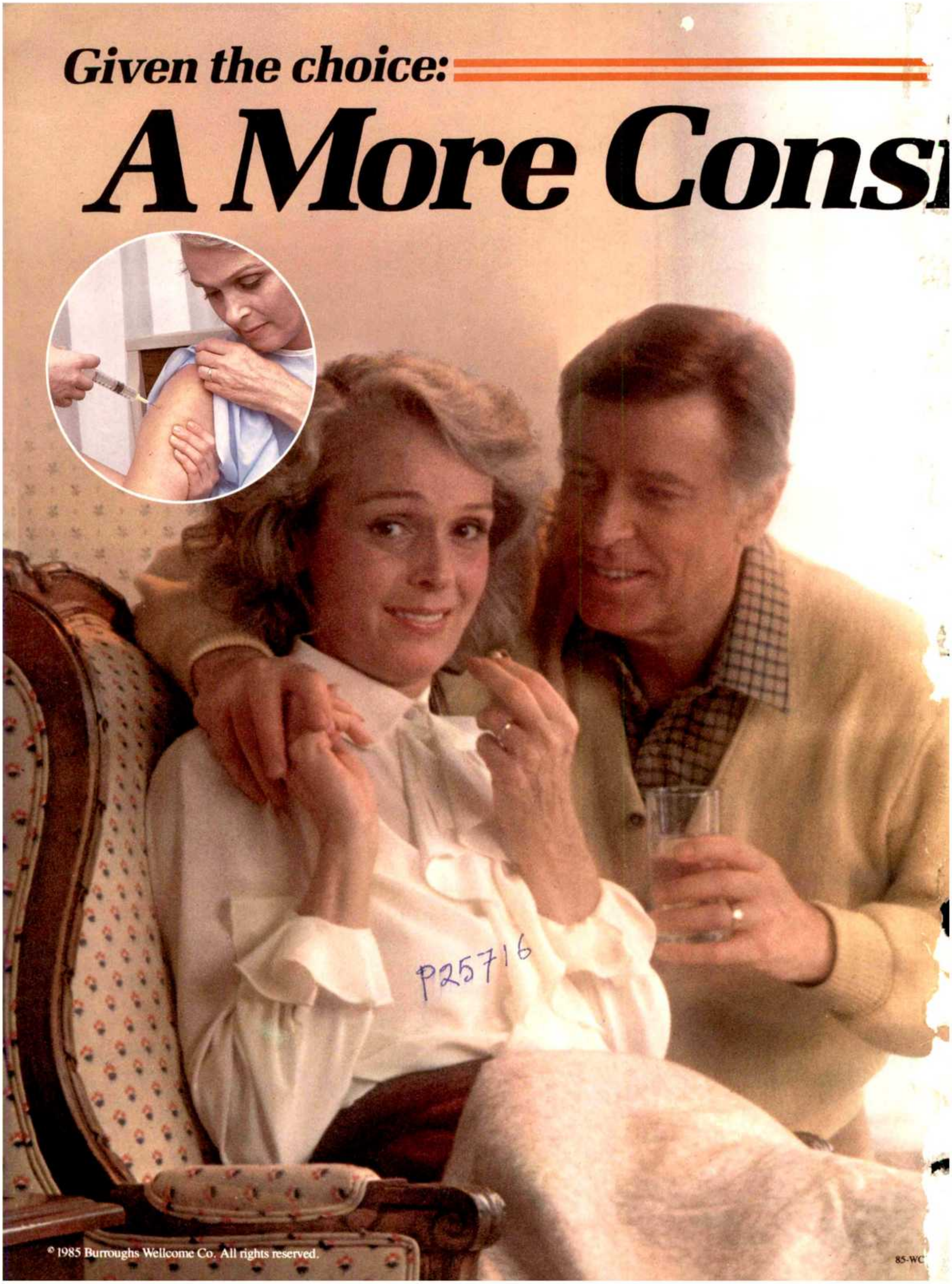

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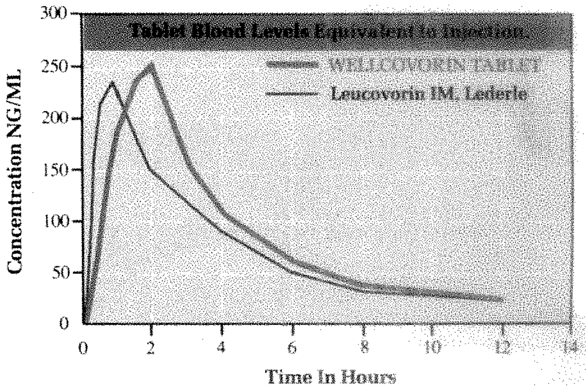
Blum MR, Guaspan A, Liao SHT, et al. Bioavailability and pharmacokinetics of oral and parenteral leucovorin. 33rd National Meeting Pharm Sci. San Diego, California. 1982, 12(2):175



Average serum folates after single 15 mg dose of leucovorin administered either IM or orally. Adapted from Blum, et al

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Please see brief summary of prescribing information on next page.

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INDICATIONS AND USAGE: Wellcovorin (leucovorin calcium) is indicated for the prophylaxis and treatment of undesired hematopoietic effects of folic acid antagonists (see WARNINGS).

CONTRAINDICATIONS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to the lack of vitamin B_{12}. A hematologic remission may occur while neurologic manifestations remain progressive.

WARNINGS: In the treatment of accidental overdosage of folic acid antagonists, leucovorin should be administered as promptly as possible. As the time interval between antifolate administration (e.g. methotrexate) and leucovorin rescue increases, leucovorin's effectiveness in counteracting hematologic toxicity diminishes.

PRECAUTIONS:

General: Following chemotherapy with folic acid antagonists, parenteral administration of leucovorin is preferable to oral dosing if there is a possibility that the patient may vomit and not absorb the leucovorin. In the presence of pernicious anemia a hematologic remission may occur while neurologic manifestations remain progressive. Leucovorin has no effect on other toxicities of methotrexate, such as the nephrotoxicity resulting from drug precipitation in the kidney.

Drug Interactions: Folic acid in large amounts may counteract the antiepileptic effect of phenobarbital, phenytoin and primidone, and increase the frequency of seizures in susceptible children.

Pregnancy: ***Teratogenic Effects:*** Pregnancy Category C. Animal reproduction studies have not been conducted with Wellcovorin. It is also not known whether Wellcovorin can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Wellcovorin should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Wellcovorin is administered to a nursing mother.

Pediatric Use: See "Drug Interactions".

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid.

OVERDOSAGE: Excessive amounts of leucovorin may nullify the chemotherapeutic effect of folic acid antagonists.

DOSAGE AND ADMINISTRATION: Leucovorin is a specific antidote for the hematopoietic toxicity of methotrexate and other strong inhibitors of the enzyme dihydrofolate reductase. Leucovorin rescue must begin within 24 hours of antifolate administration. A conventional leucovorin rescue dosage schedule is 10 mg/m² orally or parenterally followed by 10 mg/m² orally every six hours for seventy-two hours. If, however, at 24 hours following methotrexate administration the serum creatinine is 50% or greater than the pre-methotrexate serum creatinine, the leucovorin dose should be immediately increased to 100 mg/m² every three hours until the serum methotrexate level is below 5×10^{-8}M.[1,2]

The recommended dose of leucovorin to counteract hematologic toxicity from folic acid antagonists with less affinity for mammalian dihydrofolate reductase than methotrexate (i.e. trimethoprim, pyrimethamine) is substantially less and 5 to 15 mg of leucovorin per day has been recommended by some investigators.[3,4,5]

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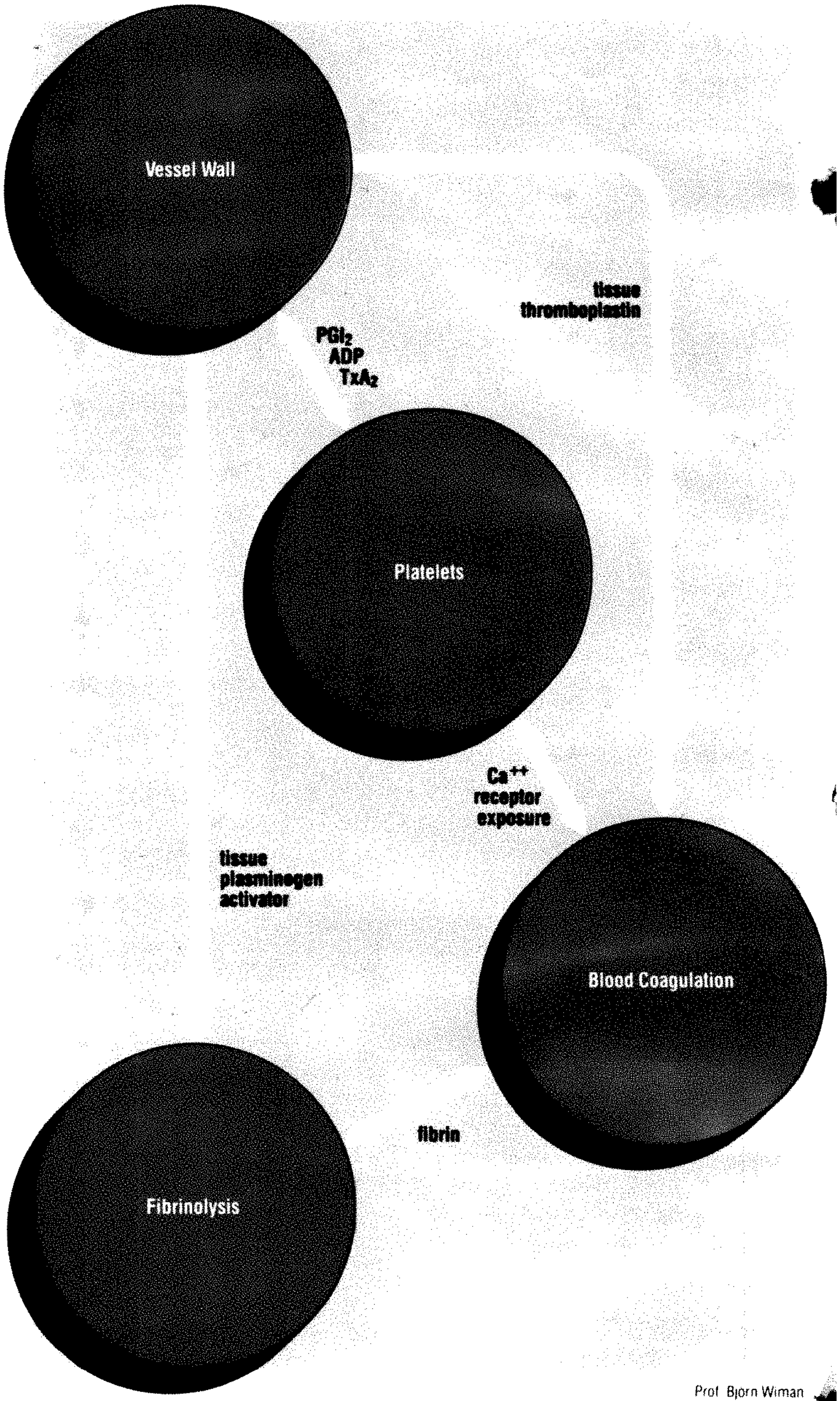

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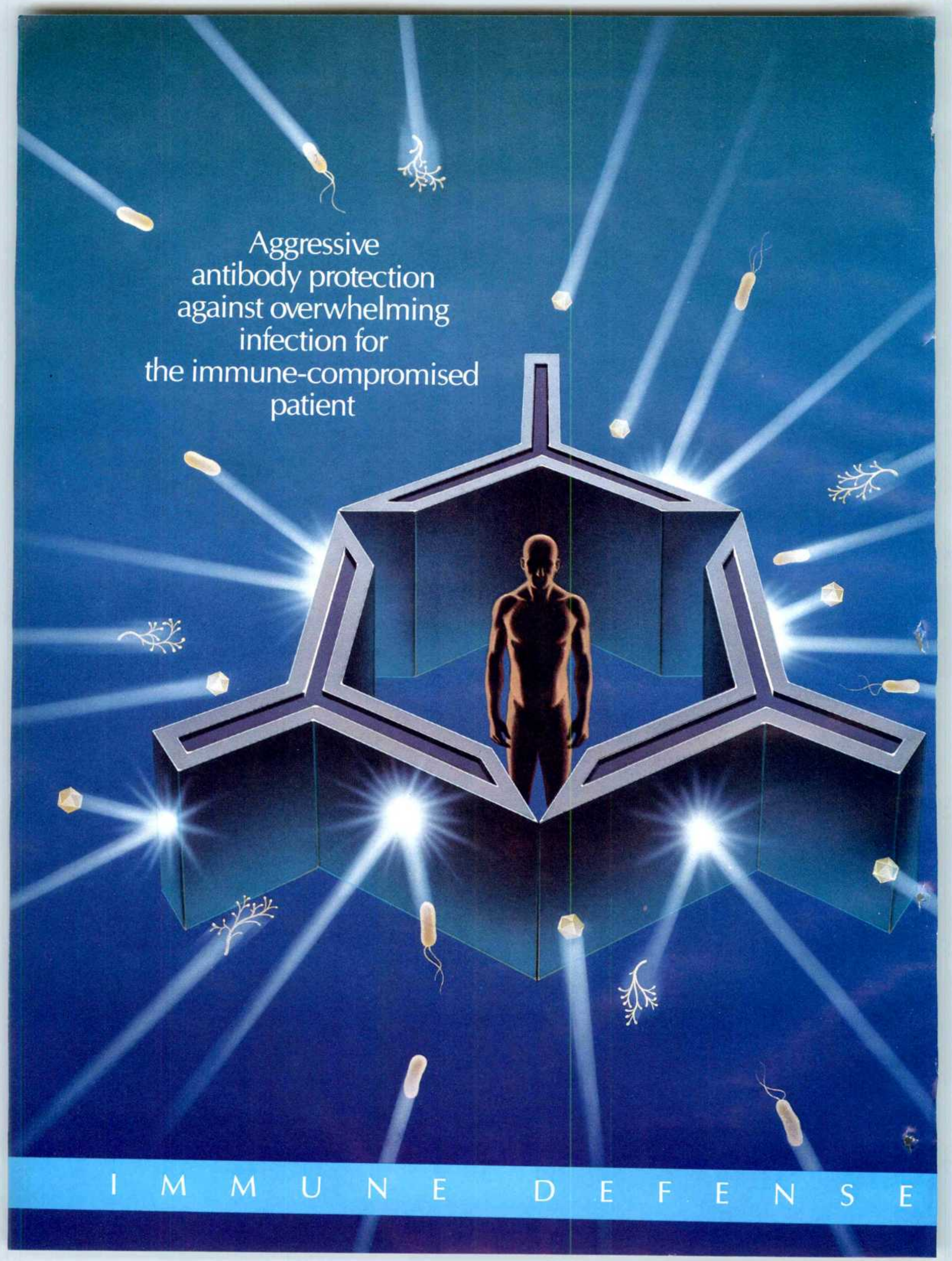
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(IFA method)	1:24	< 1:4			
			Parainfluenza		
Antistreptolysin-O	224IU	< 60IU	Type 1	1:8	< 1:8
			Type 2	1:8	< 1:8
H. influenzae Type B	1:14,700	1:1,600	Type 3	1:8	< 1:8
			Varicella-zoster	1:512	1:64
P. aeruginosa Lipopolysaccharide					
immunotype T-1	1:2,300	1:760	Cytomegalovirus		
T-2	1:7,100	1:1,070	(IFA method)	1:1,024	1:256
T-4	1:1,800	1:400	(IHA method)	1:2,048	1:512
T-6	1:1,800	1:600			
			P. carinii	1:4	< 1:4
N. meningitidis					
Type A polysaccharide	1:3,200	1:400			
Type C polysaccharide	1:8,400	1:800			
Type Y polysaccharide	1:1,400	1:200			
Type W 135 polysaccharide	1:8,400	1:1,600			

* These values represent the geometric mean titers of 5 lots of GAMIMUNE.
† These values represent a pool from at least 1,000 carefully screened donors

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Please see following page for brief summary of prescribing information.

FROM WITHIN

GAMIMUNE®

Immune Globulin Intravenous, 5% (In 10% maltose)

DESCRIPTION

Immune Globulin Intravenous, 5% in 10% Maltose (IGIV, 5%-Maltose)-Gamimune* is a sterile 5 ± 1% solution of human protein stabilized with 10 ± 2% maltose; it contains no preservatives. The immunoglobulin is selectively reduced under controlled conditions using dithiothreitol and alkylated with iodoacetamide to render it suitable for intravenous administration. Each 10 mL contains approximately 500 mg of protein, not less than 90% of which is immunoglobulin. Thus, there are approximately 450 mg of immunoglobulin in each 10 mL of IGIV, 5%-Maltose. The pH is 5.25 ± 0.25. The calculated osmolality is 327 milliosmoles per kilogram of solvent (water) and the calculated osmolarity is 292 milliosmoles per liter of solution. The product is prepared by cold alcohol fractionation from large pools of human venous plasma. Each individual unit of plasma has been tested and found nonreactive for hepatitis B surface antigen (HBsAg) using a test with third-generation sensitivity.

CLINICAL PHARMACOLOGY

Gamimune supplies IgG antibodies for prevention or attenuation of infectious diseases. The half-life of Gamimune is approximately three weeks but individual patient variation in half-life has been observed. Thus, this variable as well as the amount of immunoglobulin administered per dose is important in determining the frequency of administration of the drug for each patient.

While intravenous immunoglobulin has been shown to be effective in some cases of idiopathic thrombocytopenic purpura (ITP) (see Indications and Usage), the mechanism of action in ITP has not been fully elucidated.

Maltose is added to the solution in a 10% concentration for stabilization of the protein. A comparative clinical study of IGIV with and without maltose showed the incidence of adverse effects was significantly less with the maltose-containing preparation.

The intravenous administration of solutions of maltose has been studied by several investigators. Healthy subjects tolerated the infusions well and no adverse effects were observed at a rate of 0.25 g maltose/kg per hour. In safety studies conducted by Cutter Laboratories, infusions of 10% maltose administered at 0.27 to 0.62 g maltose/kg per hour to normal subjects produced either mild side effects (e.g., headache) or no adverse reaction. Following intravenous infusions of maltose, maltose was detected in the peripheral blood, and there was a dose dependent excretion of maltose and glucose in the urine and a mild diuretic effect. These alterations were tolerated without significant adverse effects.

Since one milliliter of Immune Globulin Intravenous, 5% in 10% Maltose (IGIV, 5% Maltose)-Gamimune* contains 0.1 g of maltose, the recommended dose of Gamimune (0.1 g to 0.4 g per kilogram body weight) would result in the patient receiving a total of 0.2 g to 0.8 g maltose per kilogram body weight given over several hours. These amounts are within those levels tolerated in the Cutter safety studies cited above.

INDICATIONS AND USAGE

Immunodeficiency Gamimune is indicated for the maintenance treatment of patients who are unable to produce sufficient amounts of IgG antibodies. Usage of Gamimune may be preferred to that of intramuscular immunoglobulin preparations especially in patients who require an immediate increase in intravascular immunoglobulin levels, in patients with a small muscle mass, and in patients with bleeding tendencies in whom intramuscular injections are contraindicated. It may be used in disease states such as congenital agammaglobulinemia (e.g., x-linked agammaglobulinemia), common variable hypogammaglobulinemia, x-linked immunodeficiency with hyper IgM, and combined immunodeficiency.

Idiopathic Thrombocytopenic Purpura Some children and adults with ITP have shown an increase (albeit temporary) in platelet counts upon administration of Gamimune. Therefore administration of Gamimune should be considered in situations that require a rapid, temporary rise in the platelet count, for example, prior to surgery or to control excessive bleeding. However, it should be noted that not all patients will respond. Even in those patients who do respond, this treatment should not be considered to be curative.

CONTRAINDICATIONS

Immune Globulin Intravenous, 5% in 10% Maltose (IGIV, 5%-Maltose)-Gamimune* is contraindicated in individuals who are known to have had an anaphylactic or severe systemic response to Immune Serum Globulin (Human). Individuals with selective IgA deficiencies who have known anti-IgA antibody should not receive IGIV, 5% Maltose since these patients may experience severe anaphylactic reactions to even small amounts of immune globulin preparations.

WARNINGS

IGIV, 5%-Maltose should be administered only intravenously as the intramuscular route has not yet been evaluated.

> IGIV, 5%-Maltose can, on occasion, cause a precipitous fall in blood pressure and the clinical picture of anaphylaxis even when the patient is not known to be sensitive to immune globulin preparations. These reactions appear to be related to the rate of infusion. Accordingly, the infusion rate given under Dosage and Administration should be closely followed, at least until the physician has had sufficient experience with a given patient. The patient's vital signs should be monitored continuously and careful observation made for any symptoms throughout the entire infusion. Epinephrine should be available for treatment of any acute anaphylactoid reaction.

PRECAUTIONS

General Any vial that has been entered should be used promptly. Partially used vials should be discarded. Do not use if turbid.

Drug Interactions If dilution is required Gamimune may be diluted with 5% dextrose. No other drug interactions or compatibilities have been evaluated. It is recommended that the infusion of Gamimune be given via separate line, by itself, without mixing with other intravenous fluids or medications the patient might be receiving.

Pregnancy Category C Animal reproduction studies have not been conducted with Immune Globulin Intravenous, 5% in 10% Maltose (IGIV, 5% Maltose)-Gamimune.* It is also not known whether Gamimune can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Gamimune should be given to a pregnant woman only if clearly needed.

Pediatric Use High dose administration of Gamimune in children with idiopathic thrombocytopenic purpura (ITP) did not reveal any adverse effects specific to the pediatric age group.

ADVERSE REACTIONS

In a small safety study of Gamimune in ten patients with primary immunodeficiency diseases, who received a total of 28 infusions at a dose of 400 mL per kg body weight, there were no adverse reactions reported during any of the infusions. Post-infusion, three patients reported adverse effects "possibly" or "definitely" related to the IGIV infusion; two patients experienced post infusion headache, and one had a flu-like syndrome consisting of joint pain, red eyes, and fatigue following the infusion.

During additional investigational studies, 17 other patients with idiopathic thrombocytopenic purpura (ITP) received a total of 148 infusions of Gamimune. Most of these infusions were at doses of 400 mg/kg body weight for 5 consecutive days; however, some patients received either 400 mg/kg or 800 mg/kg body weight as a single infusion intermittently. In these studies, adverse reactions were reported in 17 of 148 (11.5%) infusions of Gamimune. Headache, generally mild, was the most common symptom noted, occurring during or following 8% of infusions. Other less frequently reported symptoms included emesis, chills, fever, chest tightness, and nausea. With rare exceptions, symptoms were either mild or moderate in degree.

Potential reactions with administration of intravenous gamma globulins may also include anxiety, flushing, chills, wheezing, abdominal cramps, back pain, myalgia, arthralgia, malaise and dizziness; rash has been reported only rarely. These reactions tend to be related to the rate of infusion; their management is described under Dosage and Administration.

True anaphylactic reactions to IGIV, 5%-Maltose may occur in recipients with documented prior histories of severe allergic reactions to intramuscular immune serum globulin, but some patients may tolerate cautiously-administered IGIV without adverse effects. Very rarely an anaphylactoid reaction may occur in patients with no prior history of severe allergic reactions to either intramuscular or intravenous immune globulin.

DOSAGE AND ADMINISTRATION

Prophylaxis of Immunodeficiency Syndromes: The usual dose of Gamimune for prophylaxis in immunodeficiency syndromes is 100 mg/kg body weight (i.e., 2 mL/kg body weight) administered once a month by intravenous infusion. If the clinical response is inadequate or the level of IgG achieved in the circulation is felt to be insufficient, the dosage may be increased to 200 mg/kg body weight (i.e., 4 mL/kg body weight) or the infusion may be repeated more frequently than once a month.

Therapy of Idiopathic Thrombocytopenic Purpura (ITP): The recommended dosage of Gamimune for therapy of ITP is 400 mg/kg of body weight (i.e., 8 mL/kg body weight) on five consecutive days.

Administration: Recent investigations confirm that Gamimune is well tolerated and less likely to produce side effects when infused at the indicated rate. It is recommended that Gamimune be infused, by itself, at a rate of 0.01 to 0.02 mL/kg body weight per minute for 30 minutes. If the patient does not experience any discomfort, the rate may be gradually increased to between 0.02 and 0.04 mL/kg body weight per minute. If side effects occur, the rate should be reduced or the infusion interrupted until the symptoms subside. The infusion may then be resumed at a rate which is tolerated by the patient.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration whenever solution and container permit.

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Fetal Liver Transplantation

Editors
Robert Peter Gale
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HERPES SIMPLEX INFECTION IN THE COMPROMISED HOST:

OFTEN SERIOUS, TOO OFTEN UNSUSPECTED

Number one in a series of typical case presentations.

Esophagitis in a leukemia patient

In patients with hematologic malignancies, the visceral organ most frequently involved by herpes simplex virus (HSV) infection is the esophagus.[1] Extensive ulcerative lesions may develop on the upper two thirds of the esophagus with confluent lesions of the distal third.[2] In some cases, however, the mucosa may be grossly unremarkable even when microscopic examination shows evidence of herpetic ulcerative esophagitis.[1] Similarly, some patients may display symptoms of dysphagia and burning retrosternal pain,[3] whereas others may appear asymptomatic.[1] The diagnostic method of choice for patients with suspected herpetic esophagitis is esophagoscopy with visualization of the mucosa and procurement of cytologic and biopsy specimens from ulcerated areas.[1]

References: 1. Buss DH, Scharyj M: Herpesvirus infection of the esophagus and other visceral organs in adults. Incidence and clinical significance. *Am J Med* 1979;66:457-462. **2.** Wong KK, Hirsch MS: Herpes virus infections in patients with neoplastic disease. Diagnosis and therapy. *Am J Med* 1984; 76:464-478. **3.** Lightdale CJ, Wolf DJ, Marcucci RA, et al: Herpetic esophagitis in patients with cancer: Ante mortem diagnosis by brush cytology. *Cancer* 1977;39:223-226.

Wellcome

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Herpetic infections may result in extensive disease and death

Immunocompromised patients are highly vulnerable to HSV infection. Such infections may interrupt therapy and even lead to death if not detected early and managed aggressively.[1] Although the initial appearance may be mild, HSV infection may progress to severe localized mucocutaneous ulcerations. The greatest threat that herpes simplex poses is an infection that disseminates from a mucocutaneous or unrecognized primary lesion and involves the lungs, liver, esophagus, or central nervous system.[1] Furthermore, herpetic ulcers frequently provide an entry for secondary bacterial and fungal pathogens.[2]

Infections may not be diagnosed correctly without virologic study

Some investigators claim that the actual incidence of serious HSV infection is probably higher than reported because "the possibility of herpetic disease is insufficiently considered and appropriate diagnostic viral studies are often not carried out."[3] One study, for example, found histocytologic evidence of visceral herpes infection in 56 patients even though herpetic involvement was not suspected in any of the patients prior to autopsy.[2]

ZOVIRAX I.V. halts viral replication and speeds healing even in debilitated patients

ZOVIRAX I.V. has been shown to be a highly effective treatment for severe mucocutaneous HSV infection in immunocompromised patients. In controlled trials, ZOVIRAX I.V. rapidly halted viral shedding — within 3 days in most patients — and significantly shortened healing time as well as duration of pain.[4,5] Furthermore, ZOVIRAX I.V. may allow patients to return home sooner and enable patients with intraoral lesions to resume normal eating habits.[6]

ZOVIRAX I.V. is well tolerated by immunocompromised patients

A multicenter collaborative trial of ZOVIRAX I.V. in immunocompromised patients reported that: "Clinical benefits were obtained without appreciable toxicity. The most common reactions were irritation of peripheral veins used for drug infusion and a low incidence of rash.... Significant renal or hematologic toxicity did not occur, even in these seriously ill patients who were commonly receiving other drugs concomitantly."[5]

Note: Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes. Please see brief summary for more information.

References: 1. Feldman S, Cox F: Viral infections and hematological malignancies. *Clin Haematol* 1976;5:311-328. **2.** Buss DH, Scharyj M: Herpesvirus infection of the esophagus and other visceral organs in adults: Incidence and clinical significance. *Am J Med* 1979;66:457-462. **3.** Muller SA, Herrmann EC, Winkelmann RK: Herpes simplex infections in hematologic malignancies. *Am J Med* 1972;52:102-114. **4.** Wade JC, Newton B, McLaren C, et al: Intravenous acyclovir to treat mucocutaneous herpes simplex virus infection after marrow transplantation. *Ann Intern Med* 1982;96:265-269. **5.** Meyers JD, Wade JC, Mitchell CD, et al: Multicenter collaborative trial of intravenous acyclovir for treatment of mucocutaneous herpes simplex virus infection in the immunocompromised host. *Am J Med* 1982;73(1A):229-235. **6.** Data on file, Burroughs Wellcome Co.

An effective response to a life-threatening infection
ZOVIRAX® I.V. INFUSION
(acyclovir sodium) sterile powder

An effective response to a life-threatening infection

ZOVIRAX® I.V. INFUSION (acyclovir sodium) sterile powder

FOR INTRAVENOUS INFUSION ONLY

INDICATIONS AND USAGE: Zovirax Sterile Powder is indicated for the treatment of initial and recurrent mucosal and cutaneous Herpes simplex (HSV-1 and HSV-2) infections in immunocompromised adults and children. It is also indicated for severe initial clinical episodes of herpes genitalis in patients who are not immunocompromised.

These indications are based on the results of several double-blind, placebo-controlled studies which evaluated the drug's effect on virus excretion, complete healing of lesions, and relief of pain.

Herpes Simplex Infections in Immunocompromised Patients

A multicenter trial of Zovirax Sterile Powder at a dose of 250 mg/M^2 every 8 hours (750 mg/M^2/day) for 7 days was conducted in 97 immunocompromised patients with oro-facial, esophageal, genital and other localized infections (50 treated with Zovirax and 47 with placebo). Zovirax significantly decreased virus excretion, reduced pain, and promoted scabbing and rapid healing of lesions.[1,2,3]

Initial Episodes of Herpes Genitalis

A controlled trial was conducted in 28 patients with severe initial episodes of herpes genitalis with a Zovirax dosage of 5 mg/kg every 8 hours for 5 days (12 patients treated with Zovirax and 16 with placebo). Significant treatment effects were seen in elimination of virus from lesions and in reduction of healing times.[4]

In a similar study, 15 patients with initial episodes of genital herpes were treated with Zovirax 5 mg/kg every 8 hours for 5 days and 15 with placebo. Zovirax decreased the duration of viral excretion, new lesion formation, duration of vesicles and promoted more rapid healing of all lesions.[5]

Diagnosis

The use of appropriate laboratory diagnostic procedures will help to establish the etiologic diagnosis. Positive cultures for Herpes simplex virus offer a reliable means for confirmation of the diagnosis. In initial episodes of genital herpes, appropriate examinations should be performed to rule out other sexually transmitted diseases. Whereas cutaneous lesions associated with Herpes simplex infections are often characteristic, the finding of multinucleated giant cells in smears prepared from lesion exudate or scrapings may assist in the diagnosis.[6]

CONTRAINDICATIONS: Zovirax Sterile Powder is contraindicated for patients who develop hypersensitivity to the drug.

WARNINGS: Zovirax Sterile Powder is intended for intravenous infusion only, and should not be administered topically, intramuscularly, orally, subcutaneously, or in the eye. Intravenous infusions must be given over a period of at least 1 (one) hour to prevent renal tubular damage (see PRECAUTIONS and DOSAGE AND ADMINISTRATION).

PRECAUTIONS:

General: The recommended dosage, frequency and length of treatment should not be exceeded (See DOSAGE AND ADMINISTRATION).

Although the aqueous solubility of acyclovir sodium (for infusion) is >100 mg/ml, precipitation of acyclovir crystals in renal tubules can occur if the maximum solubility of free acyclovir (2.5 mg/ml at 37°C in water) is exceeded or if the drug is administered by bolus injection. This complication causes a rise in serum creatinine and blood urea nitrogen (BUN), and a decrease in renal creatinine clearance. Ensuing renal tubular damage can produce acute renal failure.

Abnormal renal function (decreased creatinine clearance) can occur as a result of acyclovir administration and depends on the state of the patient's hydration, other treatments, and the rate of drug administration. Bolus administration of the drug leads to a 10% incidence of renal dysfunction, while in controlled studies, infusion of 5 mg/kg (250 mg/M^2) over an hour was associated with a lower frequency — 4.6%. Concomitant use of other nephrotoxic drugs, pre-existing renal disease, and dehydration make further renal impairment with acyclovir more likely. In most instances, alterations of renal function were transient and resolved spontaneously or with improvement of water and electrolyte balance, drug dosage adjustment or discontinuation of drug administration. However, in some instances, these changes may progress to acute renal failure.

Administration of Zovirax by intravenous infusion must be accompanied by adequate hydration. Since maximum urine concentration occurs within the first 2 hours following infusion, particular attention should be given to establishing sufficient urine flow during that period in order to prevent precipitation in renal tubules.

When dosage adjustments are required they should be based on estimated creatinine clearance (See DOSAGE AND ADMINISTRATION).

Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes characterized by either lethargy, obtundation, tremors, confusion, hallucinations, agitation, seizures or coma. Zovirax should be used with caution in those patients who have underlying neurologic abnormalities and those with serious renal, hepatic, or electrolyte abnormalities or significant hypoxia. It should also be used with caution in patients who have manifested prior neurologic reactions to cytotoxic drugs or those receiving concomitant intrathecal methotrexate or interferon.

Exposure of HSV isolates to acyclovir *in vitro* can lead to the emergence of less sensitive viruses. These viruses usually are deficient in thymidine kinase (required for acyclovir activation) and are less pathogenic in animals. Similar isolates have been observed in 6 severely immunocompromised patients during the course of controlled and uncontrolled studies of intravenously administered Zovirax. These occurred in patients with congenital severe combined immunodeficiencies or following bone marrow transplantation. The presence of these viruses was not associated with a worsening of clinical illness and, in some instances, the virus disappeared spontaneously. The possibility of the appearance of less sensitive viruses must be borne in mind when treating such patients. The relationship between the *in vitro* sensitivity of herpesviruses to acyclovir and clinical response to therapy has yet to be established.

Drug Interactions: Co-administration of probenecid with acyclovir has been shown to increase the mean half-life and the area under the concentration-time curve. Urinary excretion and renal clearance were correspondingly reduced. Clinical experience has identified no other significant interactions resulting from administration of other drugs concomitantly with Zovirax Sterile Powder.

Carcinogenesis, Mutagenesis, Impairment of Fertility: Acyclovir was tested in lifetime bioassays in rats and mice at single daily doses of 50, 150 and 450 mg/kg given by gavage. There was no statistically significant difference in the incidence of tumors between treated and control animals, nor did acyclovir appear to shorten the latency of tumors. In 2 *in vitro* cell transformation assays, used to provide preliminary assessment of potential oncogenicity in advance of these more definitive lifetime bioassays in rodents, conflicting results were obtained. Acyclovir was positive at the highest dose used in one system and the resulting morphologically transformed cells formed tumors when inoculated into immunosuppressed, syngeneic, weanling mice. Acyclovir was negative in another transformation system.

No chromosome damage was observed at maximum tolerated parenteral doses of 100 mg/kg acyclovir in rats or Chinese hamsters; higher doses of 500 and 1000 mg/kg were clastogenic in Chinese hamsters. In addition, no activity was found in a dominant lethal study in mice. In 9 of 11 microbial and mammalian cell assays, no evidence of mutagenicity was observed. In 2 mammalian cell assays (human lymphocytes and L5178Y mouse lymphoma cells *in vitro*), positive responses for mutagenicity and chromosomal damage occurred, but only at concentrations at least 25 times the acyclovir plasma levels achieved in man.

Acyclovir does not impair fertility or reproduction in mice at oral doses up to 450 mg/kg/day. In female rabbits treated subcutaneously with acyclovir subsequent to mating, there was a statistically significant decrease in implantation efficiency but no concomitant decrease in litter size at a dose of 50 mg/kg/day.

Pregnancy: Teratogenic Effects. Pregnancy Category C. Acyclovir was not teratogenic in the mouse (450 mg/kg/day, p.o.), rabbit (50 mg/kg/day, s.c.) or rat (50 mg/kg/day, s.c.).

Although maximum tolerated doses were tested in teratology studies, the plasma levels obtained did not exaggerate maximum plasma levels that might occur with clinical use of intravenous acyclovir.

There have been no adequate and well-controlled studies in pregnant women. Acyclovir should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Zovirax is administered to a nursing woman.

ADVERSE REACTIONS: The most frequent adverse reactions reported during controlled clinical trials of Zovirax in 64 patients were inflammation or phlebitis at the injection site following infiltration of the I.V. fluid in 9 (14.0%), transient elevations of serum creatinine in 3 (4.7%), and rash or hives in 3 (4.7%). Less frequent adverse reactions were diaphoresis, hematuria, hypotension, headache and nausea, each of which occurred in 1 patient (1.6%). Of the 63 patients receiving placebo, 3 (4.8%) experienced inflammation/phlebitis and 3 (4.8%) experienced rash or itching. Hematuria and nausea were experienced by placebo recipients at the same frequency.

Among 51 immunocompromised patients, one, a bone marrow transplant recipient with pneumonitis, developed seizures, cerebral edema, coma and expired with changes consistent with cerebral anoxia on postmortem biopsy; another immunocompromised patient exhibited coarse tremor and clonus.

Additional adverse reactions were reported in uncontrolled trials. The most frequent adverse reaction was elevated serum creatinine. This occurred in 9.8 percent of patients, usually following rapid (less than 10 minutes) intravenous infusion. Less frequent adverse experiences were thrombocytosis and jitters, each in 0.4% of patients.

Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes characterized by either lethargy, obtundation, tremors, confusion, hallucinations, agitation, seizures or coma (see PRECAUTIONS).

OVERDOSAGE: Overdosage has been reported following administration of bolus injections, or inappropriately high doses, and in patients whose fluid and electrolyte balance was not properly monitored. This has resulted in elevations in BUN, serum creatinine and subsequent renal failure.

Precipitation of acyclovir in renal tubules may occur when the solubility (2.5 mg/ml) in the intratubular fluid is exceeded (see PRECAUTIONS). A six hour hemodialysis results in a 60% decrease in plasma acyclovir concentration. Data concerning peritoneal dialysis are incomplete but indicate that this method may be significantly less efficient in removing acyclovir from the blood. In the event of acute renal failure and anuria, the patient may benefit from hemodialysis until renal function is restored (see DOSAGE AND ADMINISTRATION).

DOSAGE AND ADMINISTRATION: CAUTION — RAPID OR BOLUS INTRAVENOUS AND INTRAMUSCULAR OR SUBCUTANEOUS INJECTION MUST BE AVOIDED.

Dosage: ***MUCOSAL AND CUTANEOUS HERPES SIMPLEX (HSV-1 and HSV-2) INFECTIONS IN IMMUNOCOMPROMISED PATIENTS*** — 5 mg/kg infused at a constant rate over 1 hour, every 8 hours (15 mg/kg/day) for 7 days in adult patients with normal renal function. In children under 12 years of age, more accurate dosing can be attained by infusing 250 mg/M^2 at a constant rate over 1 hour, every 8 hours (750 mg/M^2/day) for 7 days.

SEVERE INITIAL CLINICAL EPISODES OF HERPES GENITALIS — The same dose given above — administered for 5 days.

Therapy should be initiated as early as possible following onset of signs and symptoms.

PATIENTS WITH ACUTE OR CHRONIC RENAL IMPAIRMENT: Refer to DOSAGE AND ADMINISTRATION section for recommended doses, and adjust the dosing interval as indicated in the table below.

Creatinine Clearance (ml/min/1.73M^2)	Dose (mg/kg)	Dosing Interval (hours)
>50	5	8
25-50	5	12
10-25	5	24
0-10	2.5	24

Hemodialysis: For patients who require dialysis, the mean plasma half-life of acyclovir during hemodialysis is approximately 5 hours. This results in a 60% decrease in plasma concentrations following a 6 hour dialysis period. Therefore, the patient's dosing schedule should be adjusted so that a dose is administered after each dialysis.

Method of Preparation: Each 10 ml vial contains acyclovir sodium equivalent to 500 mg of acyclovir. The contents of the vial should be dissolved in 10 ml of sterile water for injection or bacteriostatic water for injection containing benzyl alcohol yielding a final concentration of 50 mg/ml of acyclovir (pH approximately 11). Shake the vial well to assure complete dissolution before measuring and transferring each individual dose. DO NOT USE BACTERIOSTATIC WATER FOR INJECTION CONTAINING PARABENS. It is incompatible with Zovirax Sterile Powder and may cause precipitation.

References: **1.** C.D. Mitchell, *et al.*, Lancet *1*(8235): 1389-1392, Jun. 27, 1981. **2.** J.C. Wade, *et al.*, Ann. Intern. Med. *96*(3): 265-269, Mar. 1982. **3.** J.D. Meyers, *et al.*, Am. J. Med. *73*(1A): 229-235, Jul. 20, 1982. **4.** Data on file, Burroughs Wellcome Co. **5.** A. Mindel, *et al.*, Lancet *1*(8274): 697-700, Mar. 27, 1982. **6.** Z.M. Naib, *et al.*, Cancer Res. *33*:1452-1463, 1973.

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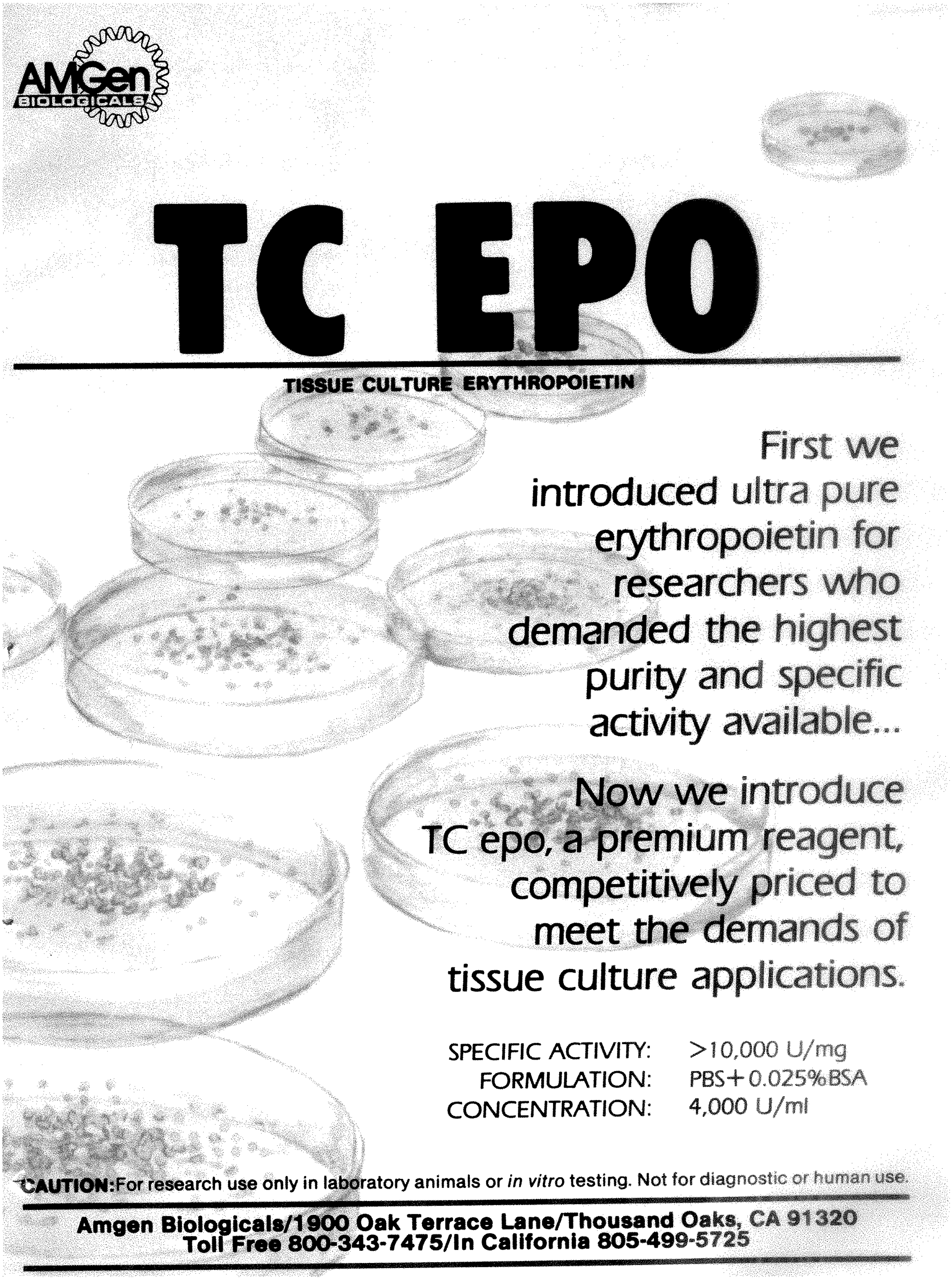

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MACOP-B

Drug Doses and Treatment Schedule for MACOP-B†

Drug	Dose	Route	Timing
Methotrexate††	400 mg/m^2	IV	Weeks 2, 6, 10
Doxorubicin	50 mg/m^2	IV	Weeks 1, 3, 5, 7, 9. 11
Cyclophosphamide	350 mg/m^2	IV	Weeks 1, 3, 5, 7, 9, 11
Vincristine	1.4 mg/m^2	IV	Weeks 2, 4, 6, 8, 10, 12
Bleomycin	10 U/m^2	IV	Weeks 4, 8, 12
Prednisone	75 mg	Oral	Daily, dose tapered over the last 15 days
Co-trimoxazole	2 tablets	Oral	Twice daily throughout

† MACOP-B = methotrexate with leucovorin rescue, doxorubicin, cyclophosphamide, vincristine, prednisone, and bleomycin; IV = intravenous.

†† Methotrexate given as a 100 mg/m^2 intravenous bolus, then 300 mg/m^2 intravenously over 4 hours, followed 24 hours later by folinic acid, 15 mg orally, every 6 hours for 6 doses.

* Klimo P, Connors JM: MACOP-B chemotherapy for the treatment of diffuse large-cell lymphoma. *Ann Intern Med.* 102:596-602, 1985.

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Brief Summary of Prescribing Information (1) 10/84. For complete prescribing information please consult product literature.

WARNING

It is recommended that Blenoxane® be administered under the supervision of a qualified physician experienced in the use of cancer chemotherapeutic agents. Appropriate management of therapy and complications is possible only when adequate diagnostic and treatment facilities are readily available.

Pulmonary fibrosis is the most severe toxicity associated with Blenoxane. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Its occurrence is higher in elderly patients and in those receiving greater than 400 units total dose, but pulmonary toxicity has been observed in young patients and those treated with low doses.

A severe idiosyncratic reaction consisting of hypotension, mental confusion, fever, chills, and wheezing has been reported in approximately 1% of lymphoma patients treated with Blenoxane.

INDICATIONS: Blenoxane should be considered a palliative treatment. It has been shown to be useful in the management of the following neoplasms either as a single agent or in proven combinations with other approved chemotherapeutic agents:

Squamous Cell Carcinoma – Head and neck including mouth, tongue, tonsil, nasopharynx, oropharynx, sinus, palate, lip, buccal mucosa, gingiva, epiglottis, skin, larynx, penis, cervix, and vulva. The response to Blenoxane is poorer in patients with head and neck cancer previously irradiated.

Lymphomas – Hodgkin's, reticulum cell sarcoma, lymphosarcoma.

Testicular Carcinoma – Embryonal cell, choriocarcinoma, and teratocarcinoma.

CONTRAINDICATIONS: Blenoxane is contraindicated in patients who have demonstrated a hypersensitive or an idiosyncratic reaction to it.

WARNINGS: Patients receiving Blenoxane must be observed carefully and frequently during and after therapy. It should be used with extreme caution in patients with significant impairment of renal function or compromised pulmonary function.

Pulmonary toxicities occur in 10% of treated patients. In approximately 1%, the nonspecific pneumonitis induced by Blenoxane progresses to pulmonary fibrosis, and death. Although this is age and dose related, the toxicity is unpredictable. Frequent roentgenograms are recommended.

Idiosyncratic reactions similar to anaphylaxis have been reported in 1% of lymphoma patients treated with Blenoxane. Since these usually occur after the first or second dose, careful monitoring is essential after these doses.

Renal or hepatic toxicity, beginning as a deterioration in renal or liver function tests, have been reported, infrequently. These toxicities may occur, however, at any time after initiation of therapy.

Usage in Pregnancy: Safe use of Blenoxane in pregnant women has not been established.

ADVERSE REACTIONS: Pulmonary – This is potentially the most serious side effect, occurring in approximately 10% of treated patients. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Approximately 1% of patients treated have died of pulmonary fibrosis. Pulmonary toxicity is both dose and age-related, being more common in patients over 70 years of age and in those receiving over 400 units total dose. This toxicity, however, is unpredictable and has been seen occasionally in young patients receiving low doses.

Because of lack of specificity of the clinical syndrome, the identification of patients with pulmonary toxicity due to Blenoxane has been extremely difficult. The earliest symptom associated with Blenoxane pulmonary toxicity is dyspnea. The earliest sign is fine rales.

Radiographically, Blenoxane-induced pneumonitis produces nonspecific patchy opacities, usually of lower lung fields. The most common changes in pulmonary function tests are a decrease in total lung volume and a decrease in vital capacity. However, these changes are not predictive of the development of pulmonary fibrosis.

The microscopic tissue changes due to Blenoxane toxicity include bronchiolar squamous metaplasia, reactive macrophages, atypical alveolar epithelial cells, fibrinous edema, and interstitial fibrosis. The acute stage may involve capillary changes and subsequent fibrinous exudation into alveoli producing a change similar to hyaline membrane formation and progressing to a diffuse interstitial fibrosis resembling the Hamman-Rich syndrome. These microscopic findings are nonspecific, e.g., similar changes are seen in radiation pneumonitis, pneumocystic pneumonitis.

To monitor the onset of pulmonary toxicity, roentgenograms of the chest should be taken every 1 to 2 weeks. If pulmonary changes are noted, treatment should be discontinued until it can be determined if they are drug related. Recent studies have suggested that sequential measurement of the pulmonary diffusion capacity for carbon monoxide (DL_{co}) during treatment with Blenoxane may be an indicator of subclinical pulmonary toxicity. It is recommended that the DL_{co} be monitored monthly if it is to be employed to detect pulmonary toxicities, and thus the drug should be discontinued when the DL_{co} falls below 30 to 35% of the pretreatment value.

Because of bleomycin's sensitization of lung tissue, patients who have received bleomycin are at greater risk of developing pulmonary toxicity when oxygen is administered at surgery. While long exposure to very high oxygen concentrations is a known cause of lung damage, after bleomycin administration, lung damage can occur at lower concentrations than usually would be considered safe. Suggested preventive measures are:

(1) Maintain FI O$_2$ at concentrations approximately that of room air (25%) during surgery and the post-operative period.

(2) Monitor carefully fluid replacement, focusing more on colloid administration rather than crystalloid.

Idiosyncratic Reactions – In approximately 1% of the lymphoma patients treated with Blenoxane an idiosyncratic reaction, similar to anaphylaxis clinically, has been reported. The reaction may be immediate or delayed for several hours, and usually occurs after the first or second dose. It consists of hypotension, mental confusion, fever, chills, and wheezing. Treatment is symptomatic including volume expansion, pressor agents, antihistamines, and corticosteroids.

Integument and Mucous Membranes – These are the most frequent side effects, being reported in approximately 50% of treated patients. These consist of erythema, rash, striae, vesiculation, hyperpigmentation, and tenderness of the skin. Hyperkeratosis, nail changes, alopecia, pruritus, and stomatitis have also been reported. It was necessary to discontinue Blenoxane therapy in 2% of treated patients because of these toxicities.

Skin toxicity is a relatively late manifestation usually developing in the 2nd and 3rd week of treatment after 150 to 200 units of Blenoxane have been administered and appears to be related to the cumulative dose.

Other – Fever, chills, and vomiting were frequently reported side effects. Anorexia and weight loss are common and may persist long after termination of the medication. Pain at tumor site, phlebitis, and other local reactions were reported infrequently.

There are isolated reports of Raynaud's phenomenon occurring in patients with testicular carcinomas treated with a combination of Blenoxane and Velban®. It is currently unknown if the cause for the Raynaud's phenomenon in these cases is the disease, Blenoxane, Velban, or a combination of any or all of these.

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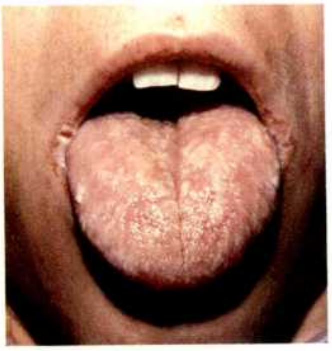
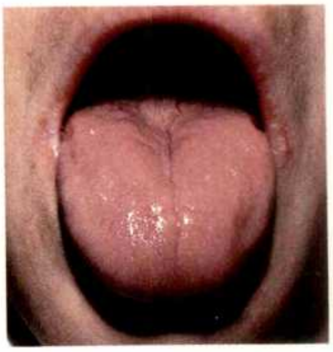


Before treatment.[1]



After treatment with MYCELEX Troche.[1]

High clinical cure* rates.

A clinical cure rate of 96% was achieved in one randomized double-blind trial[2] in which MYCELEX Troche was administered to patients with oropharyngeal candidiasis. Other investigators have concluded that MYCELEX Troche "is highly effective treatment for chronic oral candidiasis."[3]

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Patients attain a high degree of relief at the end of therapy, as evidenced by the dramatic decrease in severity of glossitis, patches, perleche, pain, and other clinical signs and symptoms of thrush.[4]

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With MYCELEX Troche, messy suspensions and lengthy swishing are things of the past. MYCELEX Troche has a pleasant, slightly sweet taste. And an entire day's therapy—5 troches—can easily be carried in pocket or purse.

Based on a study of 52 episodes of oropharyngeal candidiasis, the investigators stated: "We believe that the troche form of clotrimazole... in the treatment of thrush in any patient population... is both effective and safe."[2†]

---

*Clinical cure is defined as absence of signs and symptoms of the disease.

†**See adjacent page for summary of prescribing information, including contraindications and adverse reactions.**

Mycelex® Troche

(clotrimazole) 10 mg

The only troche for oropharyngeal candidiasis.

MYCELEX® (clotrimazole) TROCHE

Indications and Usage: Mycelex Troches are indicated for the local treatment of oropharyngeal candidiasis. The diagnosis should be confirmed by a KOH smear and/or culture prior to treatment.

Contraindications: Mycelex Troches are contraindicated in patients who are hypersensitive to any of the components.

Warning: Mycelex Troches are not indicated for the treatment of systemic mycoses.

Precautions: Abnormal liver function tests have been reported in patients treated with clotrimazole troches; elevated SGOT levels were reported in about 15% of patients in the clinical trials. In most cases the elevations were minimal and it was often impossible to distinguish effects of clotrimazole from those of other therapy and the underlying disease (malignancy in most cases). Periodic assessment of hepatic function is advisable particularly in patients with preexisting hepatic impairment.

Since patients must be instructed to allow each troche to dissolve slowly in the mouth in order to achieve maximum effect of the medication, they must be of such an age and physical and/or mental condition to comprehend such instructions.

Carcinogenesis: An 18-month dosing study with clotrimazole in rats has not revealed any carcinogenic effect.

Usage in Pregnancy: Pregnancy Category C: Clotrimazole has been shown to be embryotoxic in rats and mice when given in doses 100 times the adult human dose (in mg/kg), possibly secondary to maternal toxicity. The drug was not teratogenic in mice, rabbits, and rats when given in doses up to 200, 180, and 100 times the human dose.

Clotrimazole given orally to mice from nine weeks before mating through weaning at a dose 120 times the human dose was associated with impairment of mating, decreased number of viable young, and decreased survival to weaning. No effects were observed at 60 times the human dose. When the drug was given to rats during a similar time period at 50 times the human dose, there was a slight decrease in the number of pups per litter and decreased pup viability.

There are no adequate and well-controlled studies in pregnant women. Clotrimazole troches should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Pediatric Use: Safety and effectiveness of clotrimazole in children below the age of three years have not been established; therefore, its use in such patients is not recommended.

Adverse Reactions: Abnormal liver function tests have been reported in patients treated with clotrimazole troches; elevated SGOT levels were reported in about 15% of patients in the clinical trials (see Precautions section).

Nausea and vomiting was reported in about one in 20 patients.

Dosage and Administration: Mycelex Troches are administered only as a lozenge that must be dissolved slowly in the mouth. The recommended dose is one troche five times a day for 14 consecutive days. Only limited data are available on the safety and effectiveness of the clotrimazole troche after prolonged administration; therefore, therapy should be limited to short-term use, if possible.

How Supplied: Mycelex Troches—white, discoid, uncoated tablets are supplied in bottles of 70 and 140. Each tablet will be identified with the following: Miles 095.

Store below 86°F (30°C), avoid freezing.

References: 1. Montes LF, et al: Clotrimazole troches: a new therapeutic approach to oral candidiasis. *Cutis* 17:277-280, 1976. **2.** Yap B-S, Bodey GP: Oropharyngeal candidiasis treated with a troche form of clotrimazole. *Arch Intern Med* 139:656-657, 1979. **3.** Kirkpatrick CH, Alling DW: Treatment of chronic oral candidiasis with clotrimazole troches: a controlled clinical trial. *N Engl J Med* 299:1201-1203, 1978. **4.** Data on file, Miles Pharmaceuticals.

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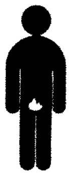
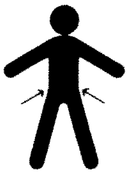


Burning or difficulty during urination

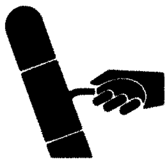
More frequent urination, particularly at night

Passage of bloody-appearing urine

Puffiness around eyes, swelling of hands and feet, especially in children



Pain in small of back just below the ribs (not aggravated by movement)



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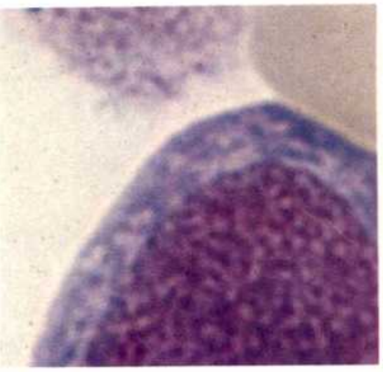

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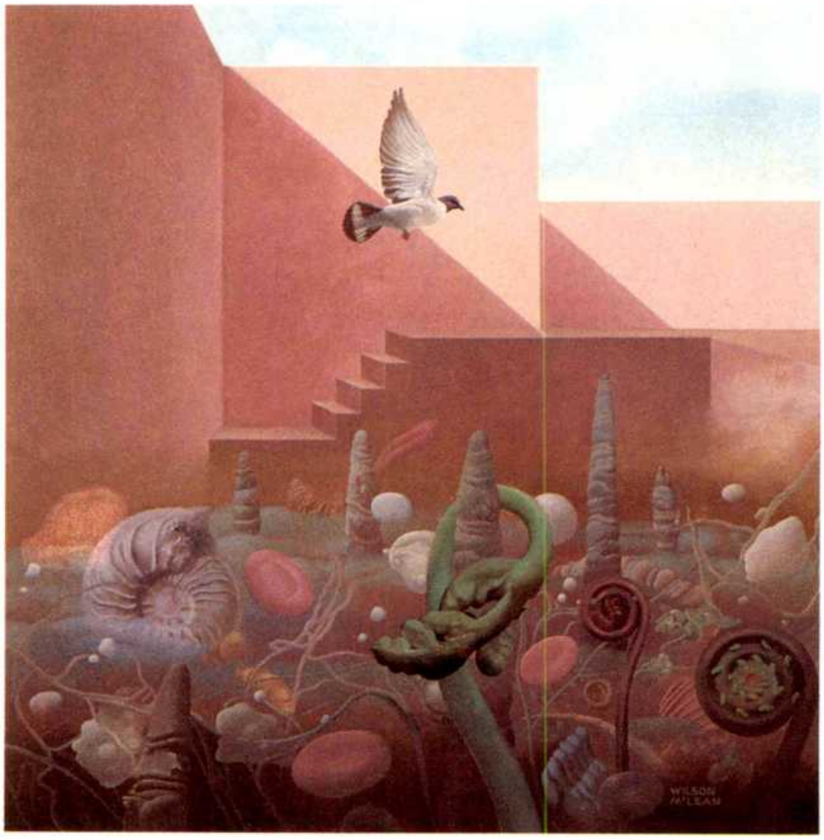

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The Journal of
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VOL 67, NO 2

FEBRUARY 1986

REVIEW

The Molecular Biology and Functions of the Granulocyte-Macrophage Colony-Stimulating Factors

By D. Metcalf

Rapid progress has occurred recently in characterizing the molecular nature of the specific glycoprotein colony-stimulating factors (CSFs) controlling the proliferation and some functional activities of granulocytes and monocyte-macrophages. All four known murine CSFs have been purified, and cDNAs for two have been cloned and expressed by mammalian and bacterial cells. Similarly, three human CSFs have been purified, and cDNAs for two cloned and expressed. This work has opened up the exciting prospects of testing the effects of these recombinant CSFs on hematopoiesis in vivo. Each CSF has a broader range of hematopoietic target cells than previously suspected, and it is now clear that the CSFs are not simply proliferative stimuli but can also regulate the functional activity of mature cells. There are increasing reasons to believe that these CSFs will be useful therapeutic agents in stimulating hematopoietic regeneration in leukopenic states and the functional activity of granulocytes and monocytes in infections.

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A MAJOR CONSEQUENCE of the development of semisolid culture systems supporting the clonal growth of hematopoietic colonies[1,2] was the recognition that hematopoietic precursor cells are unable to survive or proliferate in vitro unless specifically stimulated. This led to the discovery of a group of specific regulatory glycoproteins that stimulate cell proliferation and at least some aspects of the functional activity of these various hematopoietic subpopulations.

Since these regulatory glycoproteins were first identified because of their ability to stimulate precursor cells to form colonies of progeny cells, they are usually referred to as the colony-stimulating factors (CSFs).[3] The best characterized of these CSFs are those controlling granulocyte-macrophage (GM) populations, the granulocyte-macrophage CSFs. It is useful to comment in passing that analogous regulatory glycoproteins exist for hematopoietic cells in other lineages, bearing a variety of names For example, erythropoietin[4,5] has the typical general properties of a CSF, as does the T cell regulator, interleukin 2 (IL 2 or TCGF),[6,7] and less well-characterized regulators with the general properties of CSFs have been identified for cells of the B lymphocyte lineage (eg, BCGF)[8,9] and the eosinophil series (EDF).[10]

THE GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTORS

Four CSFs have so far been clearly identified as interacting to control the proliferation of mouse granulocytes and macrophages (Table 1). As indicated in the table, there is no uniformly accepted nomenclature for these CSFs and, because of the multiple properties of the CSFs, there are difficulties in deciding on a terminology that will be both descriptive and unambiguous. For the present, we prefer at least for the murine CSFs to use a prefix to indicate the major cell populations produced as a consequence of their action at low to moderate concentrations. Thus GM-CSF stimulates the production of both granulocytic and macrophage cells, G-CSF preferentially stimulates the formation of granulocytes and M-CSF the formation of macrophages, and Multi-CSF was chosen as an appropriate abbreviation for the molecule that has not only the capacity to stimulate granulocyte and macrophage formation but also an exceptionally broad range of proliferative effects, including actions on erythroid, megakaryocytic, eosinophil, mast, stem, and multipotential cells.

IL 3 is a term commonly used for Multi-CSF but carries with it an unsubstantiated assumption that it is an "interleukocyte" (IL) factor exclusively of leukocyte (T lymphocyte) origin and does not indicate the action of this molecule on erythroid and stem cell populations. An alternative numbering system has been advanced—so far only CSF-1 has been used extensively (for M-CSF)—but this system has the disadvantage of giving no indication of a factor's major action. It is probably premature to attempt a definitive nomenclature at this time since amino acid sequence and

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From the Cancer Research Unit, Walter and Eliza Hall Institute, Royal Melbourne Hospital, Victoria, Australia

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Submitted Sept 27, 1985, accepted Oct 25, 1985

Address reprint requests to Dr D. Metcalf, Walter and Eliza Hall Institute of Medical Research, Royal Melbourne Hospital, P O. 3050, Victoria, Australia

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Table 1. The Known Granulocyte-Macropage Colony-Stimulating Factors

Species	Name	Alternative Nomenclature	Mol Wt of Glycoprotein	Purified	cDNA Cloned
Mouse	GM-CSF	MGI-1GM[11]	23,000	Yes[12,13]	Yes[14]
	G-CSF	MGI-1G[11]	25,000	Yes[15]	No
	M-CSF	CSF-1,[16] MGI-1M[11]	40,000–70,000	Yes[17]	No
	Multi-CSF	IL 3,[18] BPA,[19] SAF,[20] HCGF,[21] PSF,[22] mast cell growth factor[23]	23,000–30,000	Yes[18,22,24]	Yes[25,26]
Human	GM-CSF[28]	NIF-T,[27] CSFα	22,000	Yes[27]	Yes[28,35]
	G-CSF	CSFβ	±30,000	No	No
	Human urine CSF		45,000	Yes[29]	Yes[30]
	Pluripoietin		18,000	Yes[31]	No

Sequence homology exists between human CSFα and murine GM-CSF,[14,28] complete functional cross-reactivity between human CSFβ and murine G-CSF,[32] and sequence homology and partial cross-reactivity between human urine CSF and murine M-CSF.[30]

membrane receptor cross-binding studies in progress may ultimately allow a rational grouping and acceptable nomenclature for these CSFs.

For human granulocytes and macrophages, four CSFs have been identified so far as being active on these populations, and these are also listed in Table 1. The general biology of these CSFs, the sites and control of CSF production, and other details have recently been reviewed extensively elsewhere.[3] Here attention will be concentrated on recent work on the molecular biology of these CSFs and certain aspects of their function.

MURINE GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR (GM-CSF)

Mouse GM-CSF able to stimulate granulocyte and/or macrophage colony formation was purified to homogeneity from mouse lung conditioned medium as a glycoprotein of mol wt 23,000.[12] Many mouse tissues contain and can synthesize GM-CSF with similar biologic properties, but these tissue-derived GM-CSFs differed widely in mol wt, from 23,000 to 200,000.[33] However, studies on the GM-CSFs semipurified from these various organ sources suggested that the differences were ascribable to a combination of technical artifacts and varying patterns of glycosylation.[33]

Based on NH_2-terminus amino acid sequence data obtained from purified lung GM-CSF,[13] oligonucleotide probes were used to isolate cDNAs for GM-CSF, initially from a library constructed from lung mRNA[14] and subsequently from a library constructed from mRNA from a concanavalin A–primed T lymphocyte clone (LB3).[34] Sequencing of these cDNA clones indicated that the mature GM-CSF polypeptide contains 124 amino acids and is of mol wt 14,138.[34] The polypeptide contains four cysteine residues, likely to be linked by mandatory disulphide bridges since mercaptoethanol destroys the biologic activity of GM-CSF. In subsequent studies, an essentially identical cDNA clone has been isolated from a mouse EL-4-derived library.[35]

Deletion and survey studies on GM-CSF cDNA clones indicated that the mRNA of GM-CSF can contain two AUG initiation codons.[34] From the amino acid sequences involved, the longer translation product could be displayed on the cell membrane, a possibility of interest in view of evidence suggesting that some cells, particularly in the marrow stroma, may control granulocyte proliferation by cell contact processes.[36,37]

Mouse genomic GM-CSF exists in single-copy form, confirming the biochemical conclusions on the essential identity of the various tissue-derived GM-CSFs, and the gene has been localized on chromosome 11.[14] The GM-CSF gene contains four exons,[38,39] but the long mRNA form appears to be initiated from a further exon and promoter located at least 10 kilobases (kb) 5′ to the main body of the gene.[38] Comparison of the nucleotide sequence of the GM-CSF gene with that of other hematopoietic growth factor genes (mouse Multi-CSF, mouse IL 2, human IL 2, and human interferon γ) has revealed a common decanucleotide (5′-GPuGPuTTPyCAPy-3′) within their respective 5′ flanking regions[38] that could be involved in some types of coordinate regulation of the production of these factors, for example, that following lectin stimulation of T lymphocytes.[40–42]

Expression of GM-CSF cDNA has been obtained using monkey COS cells[34,43] or a bacterial expression system[35] (Metcalf D, Burgess AW, Johnson GR, DeLamarter J, unpublished data). In both cases, the recombinant GM-CSF has the typical in vitro properties of native GM-CSF. These are the ability (1) to stimulate granulocyte and macrophage colony formation and, at high to very high concentrations, eosinophil, megakaryocyte, and erythroid colony formation, (2) to stimulate the proliferation of GM-CSF-dependent continuous cell lines, such as FDC-P1, and (3) to compete for receptor binding on hematopoietic cells by ^{125}I-labeled native GM-CSF. Antiserum against recombinant GM-CSF also neutralizes the biologic activity of native GM-CSF.[35]

Based on granulocyte-macrophage colony formation in bone marrow cultures, native GM-CSF has an activity of 12×10^8 U/mg[44] (50 units is the concentration stimulating half-maximal numbers of colonies to develop in cultures of mouse bone marrow cells), while bacterially synthesized recombinant GM-CSF has an activity of 5×10^8 U/mg. This indicates that for both forms of GM-CSF, 50% colony formation is stimulated by a concentration of approximately 10^{-12} mol/L. The data demonstrating the in vitro activity of nonglycosylated bacterially synthesized GM-CSF[35] confirm earlier evidence that the quite large carbohydrate moiety of the molecule is not necessary for biologic activity in vitro, a conclusion true also for the other murine CSFs.

MURINE MULTIPOTENTIAL COLONY-STIMULATING FACTOR (MULTI-CSF)

The only normal cell type so far documented as being able to synthesize Multi-CSF (also referred to as IL 3, BPA, PSF, HCGF, SAF, and mast cell growth factor) is the antigen- or mitogen-primed T lymphocyte.[3] However, Multi-CSF is also synthesized constitutively by the myelomonocytic leukemia cell line, WEHI-3B,[18,22,45] and by a T hybridoma.[46] Multi-CSF has been purified to homogeneity under this[24] and other names (IL 3,[18,47] PSF,[22]), with or without NH_2-terminus amino acid sequence analysis. Estimates of mol wt ranged from 23,000 to 30,000, possibly due to a varying carbohydrate content, and Multi-CSF stimulates half-maximal proliferative responses at 10^{-12} to 10^{-13} mol/L, according to the type of target population used.

Clones of cDNA for murine Multi-CSF (IL 3) have been isolated from a WEHI-3B-derived cDNA library[25] or a T lymphocyte–derived cDNA library.[26] Clones were identified by hybrid selection of mRNA using highly responsive continuous cell lines to detect Multi-CSF produced by oocytes injected with the selected mRNA. The mature Multi-CSF polypeptide appears to contain 140 amino acids and to have a mol wt of approximately 15,600. Like GM-CSF, the Multi-CSF polypeptide also contains four cysteine residues, probably linked by mandatory disulfide bridges.

The Multi-CSF gene exists in single-copy form and has five exons,[48,49] and linkage studies with *c-erb β*, known to be on chromosome 11, have located the Multi-CSF gene on chromosome 11,[50] the same chromosome on which the GM-CSF gene has been located. Many T lymphocyte clones have the ability to synthesize both GM-CSF and Multi-CSF, and exposure of such clones to antigens or inducers such as concanavalin A can cause a spectacular increase in transcription of both genes,[42] suggesting some type of common induction process.

A survey of different Multi-CSF cDNA clones has revealed essentially the same situation as discussed for GM-CSF. A variant of longer length, encoded by an additional exon at least 14 kb upstream of the Multi-CSF gene, has been observed, again raising the possibility of production by some cells of a longer, membrane-displayed Multi-CSF (Gough NM, King J, Metcalf D, Dunn AR, unpublished data).

Expression of Multi-CSF cDNA has been obtained using both monkey COS cells[43,51,52] and an *E. coli* expression system[53] (Metcalf D, Johnson GR, Nicola NA, unpublished data). In both cases, the recombinant Multi-CSF has the full range of in vitro properties previously noted for purified native Multi-CSF—not only the ability to stimulate granulocyte and/or macrophage colony formation but also an ability to stimulate (1) CFU-S self-generation, (2) colony formation by multipotential cells, BFU-E, CFU-E, eosinophil, and megakaryocyte progenitors, and (3) the proliferation of mast cell lines and Multi-CSF-dependent hematopoietic cell lines, such as 32D.[51-53] Recombinant Multi-CSF binds to the same hematopoietic cells as the native form and competes on an equimolar basis for receptor binding (Nicola NA, Metcalf D, unpublished data) and is neutralized by antisera prepared against purified interleukin 3 (Multi-CSF).[51]

MURINE GRANULOCYTE COLONY-STIMULATING FACTOR (G-CSF)

Murine G-CSF can be synthesized in vitro by the same wide range of tissues known to synthesize GM-CSF.[54] G-CSF has been purified to homogeneity from mouse lung conditioned medium as a glycoprotein of mol wt 25,000.[15] The molecule appears to be monomeric in form and to require internal disulfide bridges for biologic activity.

G-CSF stimulates the formation of half-maximal colony numbers at a concentration of 5×10^{-12} mol/L.[15] At low or medium concentrations, G-CSF stimulates exclusively the formation of granulocytic colonies that are characterized by their small average size, their maturity, and their relatively small total numbers.[55] Sequential observations indicated that G-CSF can initiate proliferation in many additional GM progenitor cells but fails to sustain this beyond two to four days.

G-CSF has a powerful capacity to induce terminal differentiation in WEHI-3B myelomonocytic leukemic cells,[15,56] and because of this, it was initially termed *differentiation factor* (DF).[54] This molecule needs to be distinguished from a larger molecule of mol wt 62,000, called *D-factor,* able to induce differentiation in M1 myeloid leukemia cells. D-factor has been purified from medium conditioned by L cells and Ehrlich ascites tumor cells,[57,58] and this factor has also been termed MGI-2.[11] This differentiation-inducing factor has no colony-stimulating activity for normal hematopoietic cells.

MURINE MACROPHAGE COLONY-STIMULATING FACTOR (M-CSF)

Murine M-CSF (CSF-1) has been shown to be a product of fibroblasts,[17] embryonic yolk sac,[59] whole embryo, and pregnant mouse uterus.[60] It was purified to homogeneity from L cell conditioned medium as a glycoprotein of mol wt 70,000,[17,61,62] and partial NH_2-terminus amino acid sequence data have been obtained. A varying carbohydrate content appears to be the basis for the lower mol wt forms of this CSF detected in yolk sac and pregnant mouse uterus. M-CSF stimulates half-maximal colony formation at a concentration of 5×10^{-12} to 5×10^{-13} mol/L[16,62] and differs from the other three murine CSFs in being a dimer of two equal subunits, the subunit polypeptides having a mol wt of approximately 14,000.[16] The subunits have no colony-stimulating activity and do not bind anti-M-CSF serum.

M-CSF stimulates the formation of predominantly macrophage colonies, but at all concentrations in serum-containing cultures, it also stimulates the formation of a small percentage of granulocyte-containing colonies.[3,62]

When combined with hemopoietin I (not in itself a colony-stimulating factor), M-CSF has a capacity to stimulate the formation of very large macrophage-containing colonies. Analysis has suggested that hemopoietin 1 induces the expression of M-CSF receptors on immature progenitor cells, possibly allowing responsiveness of such cells to M-CSF stimulation that is not seen when untreated cells are stimulated by M-CSF.[63]

RELATIONSHIPS BETWEEN THE MURINE CSFs

For each granulocyte-macrophage CSF there are characteristic differences in the proportions of granulocytic and macrophage colonies stimulated. Thus high concentrations of GM-CSF stimulate the formation of a high proportion of granulocyte-containing colonies, while low concentrations stimulate mainly the formation of macrophage-containing colonies.[64,65] In serum-free cultures, GM-CSF exhibits little capacity to stimulate granulocyte colony formation, but this is restored by addition of hydrocortisone.[66] G-CSF is a selective granulocyte colony stimulus at low concentrations and only at high concentrations stimulates the formation of macrophage-containing colonies.[55] For Multi-CSF, the proportions of granulocytic-containing and macrophage-containing colonies are not altered by concentration changes,[65] and with M-CSF, most colonies are composed of macrophages, although at all concentrations, a few granulocyte-containing colonies also develop.[65]

All four CSFs exhibit a capacity to stimulate the proliferation of cells in other hematopoietic lineages, but this varies widely in magnitude. At one extreme, Multi-CSF is also an effective proliferative stimulus for stem, multipotential, erythroid, eosinophil, megakaryocytic, and mast cells,[22,24,47,51–53] while at the other extreme, M-CSF exhibits no additional actions other than an ability to act in collaboration with hemopoietin I to stimulate as yet undefined progenitor cells with a high proliferative potential.[63] GM-CSF is intermediate in position. At high concentrations (>200 U/mL), it is an effective eosinophil proliferative stimulus,[55,65] and, while not supporting stem cell self-generation, at relatively low concentrations it can initiate but not sustain cell division in multipotential, erythroid, and megakaryocytic progenitors.[3] Use of very high concentrations of GM-CSF (>1,600 U/mL) has shown that this CSF is an effective proliferative stimulus for megakaryocyte and mixed erythroid colony formation (Metcalf D, Johnson GR, unpublished data). At the concentrations tested so far, G-CSF has no proliferative action on eosinophil populations or stem cells but can initiate proliferation in multipotential and erythroid progenitors, although this is a weaker action than that exhibited by GM-CSF.[55]

Care is required by using purified or single progenitor cells or clone transfer studies to establish that a particular action of a CSF is a genuine direct effect on the precursor cells involved. Certain indirect, or "cascade," effects of CSFs have been noted that can give rise to apparent ambiguities. For example, low concentrations of purified GM-CSF can stimulate CFU-E to form erythroid colonies by such an indirect action.[67] Similarly, M-CSF can stimulate macrophages to produce G-CSF, again causing misleading effects when M-CSF is used in some types of cultures.[68]

The proliferation of clones initiated by GM progenitor cells in response to stimulation by one CSF can be sustained by transfer of the clone to cultures containing one of the other three.[3,69] The most striking example of this is the almost complete cross-responsiveness of most GM precursors to both GM-CSF and Multi-CSF.[70] This overlapping control of GM progenitor cells is also demonstrable in cultures containing pairs of purified CSFs, eg, M-CSF plus G-CSF, where potentiation of both granulocytic and macrophage colony formation is observed.[3,71]

The functional overlap on bone marrow cells of the four CSFs is the more remarkable since in the case of the two whose actions are most similar—GM-CSF and Multi-CSF—there is no sequence homology between the two molecules and quite different predicted secondary structure.[14] Similarly, the sequence data for M-CSF indicate no homology with either GM-CSF or Multi-CSF, and the NH_2-terminus sequence data for G-CSF again indicate no homology with the other CSFs (Nicola NA, Simpson R, unpublished data).

MEMBRANE RECEPTORS FOR MURINE CSFs

The lack of structural homology between CSFs is substantiated by the documentation of distinct but coexpressed membrane receptors for the four CSFs.

Studies using ^{125}I-labeled M-CSF showed that a single class of specific membrane receptor existed and identified the receptor as a protein of mol wt 165,000[72,73] with receptor numbers highest on cells of the monocyte-macrophage lineage.[73–75] This receptor has been purified by affinity chromatography, and analysis has shown the receptor to be structurally related, and possibly identical, to the *c-fms* proto-oncogene product that is expressed at high levels in mature macrophages.[76] This is of considerable interest in view of the possibility of links between the CSFs and oncogenes and the previous demonstration that *v-erb* B encodes for a protein that is a truncated form of the EGF receptor.[77]

Although these studies suggested limitation of M-CSF receptors to cells of the monocyte-macrophage series and some undifferentiated cells, one report has described labeling of cells of the granulocyte series following incubation with ^{125}I M-CSF,[78] a finding in better agreement with the ability of M-CSF to support the proliferation of granulocytic clones.[69] Receptor numbers for M-CSF are relatively high (up to 50,000/cell) on some macrophage tumor cell lines, while lower numbers (3,000 to 15,000/cell) were demonstrated on mature monocyte-macrophages from various normal tissue sources.[16,74]

Use of ^{125}I G-CSF has documented the existence of a single class of specific receptor for G-CSF on the membranes of all murine granulocytic cells, receptor density increasing as the cells mature to postmitotic polymorphs.[79] Receptors were also demonstrable on some cells in the monocytic lineage but not on eosinophil, erythroid, or lymphoid cells. Average receptor numbers were 50 to 500/cell, and, with an apparent dissociation constant for binding of 60 to 80 pmol/L, G-CSF appears to be able to exert half-maximal proliferative effects on responding cells at low receptor occupancy. The G-CSF receptor is a monomer of mol wt 150,000 (Nicola NA, Peterson L, unpublished data).

Murine G-CSF binds with equal efficiency to human granulocytic cells, although with human cells the immature promyelocytes and myelocytes exhibit the highest numbers of receptors.[32] Competition studies suggested that the human receptors binding G-CSF were likely to be receptors for human CSFβ.

Studies using ^{125}I-labeled GM-CSF have demonstrated the presence on normal marrow cells of both high- and low-affinity receptors (average number 70/cell and 350/cell, respectively).[80] All granulocytes, monocytes, and eosinophils bear receptors for GM-CSF, the numbers decreasing with increasing cellular maturation and with mature eosinophils exhibiting half the receptor numbers of neutrophils. No receptors were noted on lymphoid or erythroid cells (Metcalf D, Walker F, Burgess AW, unpublished data). A single molecular species of receptor has been observed of mol wt 51,000.[80]

High numbers of receptors for Multi-CSF (IL 3) of a single class have been observed on continuous hematopoietic cell lines dependent on Multi-CSF (1,000–5,000/cell)[81] (Nicola NA, Peterson L, unpublished data). Lower receptor numbers have been observed on normal marrow cells (50–1,000/cell), and in autoradiographs all granulocytic, monocytic, and eosinophil cells were labeled, with receptor numbers decreasing with increasing maturation and with eosinophils exhibiting twice the labeling of neutrophils. No labeling of lymphoid or nucleated erythroid cells was observed (Metcalf D, Nicola NA, unpublished data). Multi-CSF receptors of both mol wt 55,000 and mol wt 75,000 have been observed.

It appears that most murine granulocyte-macrophage cells simultaneously exhibit receptors for three or four CSFs, an apparently redundant situation whose purpose is not clear. Recent studies have indicated intriguing cross-influences between the different CSF receptors. There is no direct competition for receptor binding between the CSFs, but binding of Multi-CSF to its receptors leads to down-modulation of receptors for all other CSFs; binding of GM-CSF to its receptors does not influence Multi-CSF receptor binding but down-modulates receptors for G-CSF and M-CSF; high concentrations of M-CSF down-modulate GM-CSF receptors; and high concentrations of G-CSF down-modulate receptors for M-CSF.[65]

HUMAN GRANULOCYTE-MACROPHAGE CSFs

Studies have identified at least two biochemically separable forms of CSF produced by various human cells that are able to stimulate granulocyte-macrophage colony formation by human cells.[32,82–84] From their sequence of elution from hydrophobic columns, these were labeled α (nonbinding) and β (binding).[82] CSFα preferentially stimulates day 14 colonies composed mainly of monocytes and macrophages, while CSFβ stimulates the formation mainly of day 7 colonies, a high proportion of which are granulocytic in composition. CSFα, but not CSFβ, also stimulates eosinophil colony formation.

Human granulocyte-macrophage CSF (CSFα). Gasson et al[27] reported the purification to homogeneity of a granulocyte-macrophage CSF from medium conditioned by the Mo hairy T leukemia cell line. This was a glycoprotein of mol wt 22,000.

Clones of cDNA for human GM-CSF have been isolated by direct expression screening of cDNA libraries from mRNA from Mo leukemic cells[28] or a human T lymphocyte cell line.[85] Transfection of these clones to monkey COS cells led to the production of a CSF able to stimulate granulocyte-macrophage colony formation by human cells. Sequencing of these cDNA clones indicated that the mature GM-CSF polypeptide contains 127 amino acids (mol wt ± 14,000) and includes four cysteine residues. In the protein-coding region there is 70% nucleotide homology with the equivalent region of murine GM-CSF, including conservation of the four cysteine residues, but there is no species cross-reactivity between murine and human GM-CSF. From the identity of deduced and observed NH_2-terminus sequence data, both cDNA clones code for the same GM-CSF as purified from Mo cell conditioned medium.[27]

The recombinant CSF produced by transfected COS cells was purified to homogeneity and had mol wt 19,000 and a specific activity of 4×10^7 U/mg protein.[28,86] Analysis of the biologic activities of recombinant CSF showed that it stimulates granulocyte and/or macrophage colony formation by human cells, colony numbers being highest at day 14 of incubation, at which time most colonies were monocyte-macrophage in composition.[85,86] Recombinant CSF is also an effective proliferative stimulus for eosinophil colony formation.[85,86] These actions are due to a direct action of the CSF on responding progenitor cells.[86] Recombinant GM-CSF is also able, via mainly indirect action, to potentiate erythroid and multipotential (GEMM) colony formation in combination with erythropoietin and is able to stimulate the functional activity of both neutrophils and eosinophils. Based on these properties, this recombinant GM-CSF has been identified as CSFα.[86]

Human CSFβ. Human CSFβ has not been purified to homogeneity but is a preferential stimulus for relatively mature granulocytic progenitors (promyelocytes and myelocytes)[87] and stimulates the formation of comparable murine granulocytic colonies. It has the size and hydrophobic properties of murine G-CSF, exhibits a capacity to induce differentiation in murine WEHI-3B leukemic cells equivalent to that of murine G-CSF, and competes at equivalent biologic concentrations with binding of ^{125}I G-CSF to either murine or human cells.[32] Based on these properties, it is likely to be a close analogue of murine G-CSF.

Human pluripoietin. The purification to homogeneity has been reported of a CSF from medium conditioned by the human bladder cancer cell line 5637.[31] This CSF, mol wt 18,000, was reported to have the ability to stimulate granulocyte and macrophage colony formation, to potentiate erythroid and multipotential colony formation by human cells, and to induce differentiation in HL60 and murine WEHI-3B colonies. These properties appear to be a combination of those exhibited by CSFα and β.

Human urinary CSF. CSF in human urine, active in stimulating granulocyte-macrophage colony formation by murine cells, was the first CSF to be purified to homogeneity.[29] This CSF is a glycoprotein of mol wt 45,000, and while it has little direct proliferative effect on human GM progenitor cells, it was shown to be able to stimulate human granulocyte-macrophage colony formation indirectly, probably by stimulating adherent accessory cells to produce a human-active CSF.[88,89]

Use of anti–murine M-CSF sera and competitive binding studies using ^{125}I M-CSF indicated that the human molecule was likely to be similar to mouse M-CSF,[90] and this has been confirmed by the demonstration that human urinary CSF, like M-CSF, is a dimer with two equal subunits,[90] with NH_2-terminus sequence data indicating homology with murine M-CSF.[30]

A cDNA for a human CSF has been isolated indirectly using nucleotide probes based on NH_2-terminus sequence for human urinary CSF.[30] These probes were used to isolate a genomic clone, and with this probe a cDNA clone was isolated from a library constructed from mRNA from a human pancreatic cancer cell line known to be able to secrete CSF. The COS cell expression product of this clone stimulates macrophage colony formation by mouse bone marrow cells and is neutralized by anti–human urine CSF serum.

GENERAL COMMENTS ON THE STRUCTURE OF THE CSFs

The CSFs show no sequence homology with any other known proteins, growth factors, or oncogenes. This structural uniqueness of the CSFs is substantiated by two biologic facts: (1) The CSFs are the only agents able to stimulate directly the proliferation of granulocyte-macrophage populations. None of the known growth factors have direct proliferative effects on GM cells, and even where agents such as the phorbol esters have been shown to stimulate colony formation,[91,92] analysis has proved this to be an indirect effect mediated by stimulation of monocyte-macrophages in the culture to produce an active CSF.[93] (2) A wide range of polypeptide growth factors have been shown to be unable to compete with CSFs for binding to their membrane receptors.

The sequence dissimilarity between the CSFs within one species indicates that although these molecules have highly specific biologic activity, often of a similar nature, they are not derivatives of some common evolutionary ancestral regulatory molecule.

MULTIPLE FUNCTIONS OF THE CSFs

Although the GM colony-stimulating factors were identified on the basis of their ability to stimulate the clonal proliferation of hematopoietic precursor cells, each exhibits three other functional activities: (1) They are necessary for, or promote, cell survival in vitro of both progenitor and mature end cells,[94,95] (2) they can commit bipotential GM progenitors to a restricted differentiation pathway of granulocyte or macrophage formation, an irreversible asymmetric process requiring passage of the cells through one or two cell divisions in the presence of the committing CSF,[69,71] and (3) they can stimulate a number of functional aspects of mature granulocytes, eosinophils, and macrophages. These effects include survival, cell mobility, shape, phagocytic activity, synthesis of biologically active molecules, antibody-dependent cytotoxicity, autofluorescence, and expression of various membrane markers.[3,27,88,95–108]

While the CSFs are not the only agents able to stimulate end cell functional activity, their action is rapid and readily measurable and can often be demonstrated using CSF concentrations lower than those required for proliferative stimulation. The CSFs should not, therefore, be viewed solely as proliferative agents but also as being capable of stimulating significant changes in the functional activity of existing mature cells.

THE CSFs, ONCOGENES, AUTOCRINE GROWTH, AND MYELOID LEUKEMIA

Since the CSFs are the only known proliferative regulators for granulocytic and macrophage cells, it has been logical to raise certain questions with respect to the CSFs and myeloid leukemia: (1) Are the genes for CSF or CSF receptors oncogenes? (2) Does autocrine production of CSF lead to myeloid leukemia? (3) Can myeloid leukemias be suppressed by manipulation of CSF levels?

There is no sequence homology between the CSFs and known oncogenes, but the list of known oncogenes is probably incomplete, particularly in respect to hematopoietic neoplasms, because of the overuse of a fibroblast (NIH 3T3) detection system. One striking observation, however, has been the demonstration of homology between the M-CSF (CSF-1) receptor and the translation product of *c-fms*,[76] *v-fms* being the viral oncogene of the McDonough strain of feline sarcoma virus.

The possible role of CSFs as autocrine stimulators of the proliferation in myeloid leukemia cells is a complex question on which available evidence is quite conflicting. A striking feature of *primary* myeloid leukemias in man and mouse (and here the situation with long-passaged myeloid leukemias needs to be discounted) is that the leukemic cells remain *absolutely* dependent on exogenous CSF for survival and continued proliferation in vitro (see review[3]). This situation persists throughout the clinical course of the disease. The data on the dependency of myeloid leukemia cells on extrinsic CSF are quite unequivocal and would lead to the firm conclusion that myeloid leukemias are unlikely to be examples of autocrine growth, given only that the in vitro data reliably reflect the situation in vivo. It must also be emphasized that in vivo there are multiple normal-tissue sources of CSF, producing CSF levels greatly in excess of those conceivably able to be produced by the first emerging leukemic cell. Whether or not the first leukemic cell produces CSF is likely to be irrelevant if it is bathed in fluid containing CSF.

Leukemic cells, both primary and passaged, can often be shown to synthesize CSF. In primary myeloid human leukemias, this is most clearly demonstrable for the mature monocytes of the leukemic clone, but most cultured cell lines of monocytic leukemias can also produce one or another CSF, and here undifferentiated cells are likely to be the cellular source of the CSF[109,110] (see review[3]). Two comments need to be made in assessing the significance of this production of CSF by leukemic cells: (1) There has been no evidence so far that the leukemic cells produce abnormal forms of CSFs to which leukemic cells are abnormally or selectively responsive. Where such CSFs have been biochemically characterized, they are demonstrable as being similar to the normal equivalent molecule. (2) At least some normal hematopoietic cells can also synthesize CSF, and the levels of CSF produced on a per cell basis are equivalent to those produced by leukemic cells.

Despite the strong circumstantial evidence against ascribing myeloid leukemia to autocrine CSF production, certain observations have linked acquisition of constitutive CSF production with leukemic transformation. Two groups studying the behavior of murine nonleukemic but immortalized hematopoietic cells that are dependent on extrinsic Multi-CSF for survival and proliferation noted that the emergence of subclones capable of autonomous proliferation and Multi-CSF production coincided with the acquisition by these cells of a capacity to produce transplanted leukemias in syngeneic recipients.[111,112] Similarly, infection of cells of one such line (dependent on Multi-CSF or GM-CSF) with a retroviral construct containing the GM-CSF cDNA led to the emergence of autonomous, GM-CSF-producing sublines that were uniformly leukemogenic on transplantation to syngeneic recipients.[113] In a similar vein, a second infection of *v-myc*-immortalized chicken macrophages with the oncogene *v-mil* resulted in the emergence of leukemic cells whose continued proliferation was dependent on the acquired synthesis and secretion of an avian M-CSF.[114] These observations make a strong case implicating constitutive and possibly dysregulated CSF synthesis as being capable of transforming immortalized cells to leukemic cells.

However, in keeping with the contradictory nature of present evidence on this question, it has been shown that Abelson virus infection of immortalized CSF-dependent murine hematopoietic cell lines transformed the cells to leukemic cells under conditions where autocrine production of either Multi-CSF or GM-CSF could be excluded and in which membrane receptors for CSF appeared to be unaltered in the transformed cells.[115,116]

Thus dysregulated CSF production by preleukemic cells may be able to lead to the transformation of such cells to fully leukemic cells, but leukemic transformation can also occur by other mechanisms, and in human myeloid leukemia development, there is still no substantial evidence for autocrine stimulation by CSF as a crucial factor in leukemia development.

Because primary myeloid leukemias are uniformly dependent on CSF for proliferation, it should be possible to suppress such leukemias by suppressing CSF levels. Unfortunately, this is not a feasible approach, since normal GM cells require stimulation by similar concentrations of CSF. However, because the CSFs can induce differentiation commitment in granulocyte-macrophage precursor cells, an intriguing alternative approach to leukemia control has emerged. With the murine WEHI-3B leukemia model, the known CSFs have been tested for their inability to induce differentiation commitment and to suppress leukemic stem cell self-generation. Of the four known CSFs, M-CSF and Multi-CSF are inactive,[3] and GM-CSF has detectable but relatively weak activity,[117] whereas G-CSF is a powerful inducer of differentiation.[15,56] At the same concentrations used to stimulate the proliferation of normal GM cells, G-CSF can reduce leukemic stem cell self-generation, induce the formation of end cell granulocytes and monocytes, and, on continued exposure, completely suppress a leukemic population.[56,118]

Analysis of these events[119] indicated a close similarity with the irreversible, asymmetric cell cycle-dependent events during commitment of normal GM progenitor cells by CSF when these cells enter a restricted differentiation pathway.[69,71]

Primary human myeloid leukemia populations express membrane receptors for G-CSF (presumably CSFβ receptors),[32] and while the CSFs are not the only biologically derived macromolecules able to induce terminal differentiation in myeloid leukemic cells,[11,57,58,120,121] it remains possible that the CSFs will be of value in the suppression of myeloid leukemias.

IN VIVO ACTIONS OF THE CSFs

A major criticism of past work on the CSFs has been that the effects of these regulators had been analyzed only in vitro. This situation arose because of the minute amounts of CSF produced by tissues or cell lines, the short in vivo half-lifes of the CSFs, and a proper reluctance to use crude CSF-containing materials to establish likely in vivo effects of injected CSF. Indirect evidence for CSF action in vivo has come from an analysis of the effects of CSF-producing tumors on host hematopoietic populations, but while these are in line with the predicted effects of CSF,[122–133] it cannot be eliminated that the effects were due to some other products of the tumor cells or to indirect responses on the part of the host to the presence of the tumor tissue

Moderate stimulating effects on granulocyte and monocyte formation were observed in studies on normal adult mice injected with partially purified CSF from human urine,[134,135] and purified CSF from this source[88] injected into leukopenic patients caused a slightly accelerated recovery of leukocyte levels.[136]

With the development of recombinant bacterially synthesized CSFs with full in vitro biologic activity, the situation has now changed. There are no publications yet on this subject, so the material cannot be formally reviewed, but data from this and other laboratories indicate that there are demonstrable effects of injected GM-CSF and Multi-CSF on white cell levels, organ cellularity, and content of progenitor cells and marked elevations of peritoneal cell levels if, in the latter instance, the intraperitoneal route of injection is used.

This comment on unpublished data is inserted here mainly to raise a question that most workers in the field have so far not properly considered. The CSFs were detected as proliferative stimuli, and most thinking on likely in vivo uses for the

CSFs has therefore involved questions of stimulation of hematopoietic regeneration following irradiation, cytotoxic drugs, and other treatments. This may or may not turn out to be the most effective application of the CSFs.

Since the CSFs have rapid stimulating effects on mature-cell functional activity, this raises the alternative possibility that a more common use of such agents will be to stimulate the functional activity of preexisting cells in situations such as acute infections, either local or systemic. It is in this context that the dramatic effects of locally injected recombinant CSF on peritoneal populations are of considerable interest.

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BRIEF REVIEW

The Control of Complement Activation by the Blood Cells in Paroxysmal Nocturnal Hemoglobinuria

By Wendell F. Rosse

SINCE THE CLASSIC studies of Ham and Dingle,[1] it has been known that the red blood cells of patients with paroxysmal nocturnal hemoglobinuria (PNH) are unusually susceptible to the hemolytic action of complement. There are two major types of abnormal cells in this syndrome: the PNH II red blood cells are moderately sensitive to the lytic action of complement (three to six times more sensitive than normal red blood cells),[2] and the PNH III red blood cells are markedly sensitive to complement (15 to 25 times more sensitive than normal cells).[3,4] The platelets[5,6] and granulocytes[5,7] are also more sensitive although the degree of abnormality is less well defined than for red cells.

The reason for this unusual susceptibility has remained elusive. The increased lysis could not be attributed to increased binding of antibody or to increased activation of any of the first three components.[8] It has been demonstrated that when complement is activated on the surface of both varieties of abnormal red cells,[8,9] platelets,[6] and granulocytes,[7] an abnormally large amount of C3 is deposited on the membrane. For red cells, this component is deposited primarily on the glycoproteins of the membrane,[10] and although these may be abnormal, they do not provide an acceptor surface that attracts activated C3b abnormally.

The activation of complement on the surface of red blood cells is regulated by a series of reactions[11] that tend to inactivate the complexes formed by that activation. One such inactivation reaction is due to the presence of a glycoprotein, decay-accelerating factor (DAF), on the surface of the red cell[12,13] that either prevents the formation of the C3 convertase complexes (C4b2a and C3bBb) or facilitates their disruption and thus their inactivation. Recently, it has been shown that DAF is absent on both PNH II and PNH III red cells as well as platelets and granulocytes in PNH.[14-17] This defect probably accounts for the increased binding of C3 since inhibition of the activity of DAF by antibody results in a marked increase in binding of C3 as is seen in PNH II and PNH III cells.[18] The deficiency of DAF appears to be acquired during maturation of the red cells since it apparently is not deficient on the surface of early red cell precursors.[19]

But does the deficiency in DAF fully explain the complement sensitivity of the abnormal PNH cells? The answer appears to be both yes and no. When normal red cells are treated with antibodies to DAF, they have the complement lysis sensitivity of PNH II cells (a three- to fivefold increase in sensitivity). When DAF is added back into the membrane of PNH II cells, they are no longer susceptible to the lytic action of complement when activated by acidification of serum.[20] Thus, the deficiency in DAF appears to account fully for the increased susceptibility of PNH II cells to lysis by complement.

PNH III cells appear to bear a second defect. Normal red cells appear to be able to modulate the fixation or insertion of the terminal lytic complex of complement consisting of C5b-9.[21] This appears to be lacking in the markedly abnormal PNH III cells since they readily fix more terminal complexes, particularly when complement is activated in fluid phase (reactive lysis).[22] These complexes are also more effective in lysing these abnormal cells.[23,24] The defect is not directly related to the deficiency of DAF since both PNH II and PNH III cells lack DAF, but only PNH III cells have this defect.

This latter defect in regulation of the effectiveness of the terminal complex is probably the more important in causing the hemolysis observed in PNH. The life span of PNH II cells measured by diisopropyl fluorophosphate (DFP)[32] is only somewhat shorter than that of normal cells, whereas the life span of the more abnormal PNH III red cells is markedly shortened.[25] Patients with only PNH II cells have little clinical hemolysis.[4] Since PNH III cells are susceptible to lysis by reactive lysis reactions and PNH II cells are not, it is likely that the majority of lysis that is observed clinically is due to activation of complement in the fluid phase or at sites external to the red cell; these reactions can generate C5b-7, which is able to bind more readily to PNH III than to PNH II or normal red cells, but this increased binding is not related to the deficiency of DAF on these cells. Further, the platelets in PNH appear to have the DAF defect[16,17] but not the terminal complex defect[26]; they appear to have a normal life span both as measured by Cr^{51} [27] and by In^{III}.[28] Thus, the deficiency of DAF appears to have relatively little effect on the survival of the affected blood cells.

What is the defect in PNH? The answer is that there is no single defect. In addition to the abnormalities outlined before that relate to the activation of complement, the abnormal cells are also known to be deficient in acetylcholinesterase.[29-31] Glycophorin α, the major glycoprotein of the red cell's surface, is also abnormal.[10] Thus, the defects that result in the unusual susceptibility to complement are part of a constellation of membrane abnormalities that are probably the result of marrow injury and dyshematopoiesis. How they are interrelated will be the subject of considerable research.

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Association of the Translocation (15;17) With Malignant Proliferation of Promyelocytes in Acute Leukemia and Chronic Myelogenous Leukemia at Blastic Crisis

By Shinichi Misawa, Edward Lee, Charles A. Schiffer, Zemin Liu, and Joseph R. Testa

Cytogenetic studies were performed on nine patients with acute promyelocytic leukemia. Every patient had an identical translocation (15;17) or, in one case, a variant three-way rearrangement between chromosomes 7, 15, and 17. Another patient with chronic myelogenous leukemia was examined at the time of blastic crisis when the patient's bone marrow was infiltrated by hypergranular promyelocytes and blasts. Bone marrow cells contained a t(15;17) as well as a Ph^1 chromosome. Only the latter abnormality was observed in the chronic phase of the disease. The translocation (15;17) was detected in all ten patients when bone marrow or peripheral blood cells were cultured for 24 hours prior to making chromosome preparations. However, the t(15;17) was not seen in three of these same cases when bone marrow cells were processed directly. These findings indicate that the t(15;17) is closely associated with acute proliferation of leukemic promyelocytes and that detection of this karyotypic defect may be influenced by the particular cytogenetic processing method used in different laboratories. An analysis of the banding pattern in the variant translocation provided additional evidence favoring chromosomal breakpoints at or very near the junction between bands 17q12 and 17q21 and at 15q22.

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SINCE THE FIRST REPORT of a specific translocation (15;17) in acute promyelocytic leukemia (APL),[1] this chromosomal rearrangement has been consistently associated with this type of acute leukemia and its microgranular variant (M3 and M3V, respectively, according to the French-American-British [FAB] classification[2,3]). Among 61 patients with de novo APL evaluated at the Fourth International Workshop on Chromosomes in Leukemia, 43 (71%) had a translocation (15;17).[4] Furthermore, the t(15;17) has not been observed in any patients with morphologic types of acute leukemia other than APL or variant APL.[4] Moreover, several investigators have recently suggested that this abnormality can be found in every patient with APL if optimal processing techniques are used.[5,6] If this is the case, the t(15;17) would represent the most specific chromosome abnormality for a particular morphologic type of leukemia.

We report here cytogenetic and clinical findings on nine consecutive patients with APL, each of whom had a t(15;17) or, in one patient, a variant form of this rearrangement. A tenth patient with Ph^1-positive chronic myelogenous leukemia (CML) was also examined at the time when this patient underwent a promyelocytic blastic crisis. In the latter case a t(15;17) was superimposed upon the Ph^1-positive cell line.

MATERIALS AND METHODS

Between Nov 1980 and June 1984 cytogenetic studies were performed on nine patients who had a diagnosis of APL.[2] Another patient who had Ph^1-positive CML of 25 months' duration was examined at the time of blastic crisis when the bone marrow was infiltrated by hypergranular promyelocytes (34%) and blasts (24%) (Table 1).

Aspirated bone marrow was examined in seven patients and unstimulated peripheral blood was studied in three others (Table 2). The cells were suspended in complete medium (RPMI 1640 with 15% fetal bovine serum, 2mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin) at a concentration of 1×10^6 cells per mL. In each case one culture was maintained at 37 °C in humidified air containing 5% CO_2 for 24 hours and then exposed to Colcemid (0.06 μg/mL) for ten minutes prior to harvest. In four patients (cases 242, 282, 303, and 580) we also examined cell cultures that were synchronized with methotrexate.[7] In three patients (cases 181, 242, and 552) a portion of the bone marrow specimen was processed by the direct technique. Chromosomes were examined with the quinacrine (Q) or Giemsa (G) banding techniques. Karyotypes are expressed according to the International System for Human Cytogenetic Nomenclature, 1978.[8] All patients in this study were advised of procedures and attendant risks in accordance with institutional guidelines and gave informed consent.

RESULTS

Eight patients with APL showed a specific translocation between chromosomes 15 and 17, t(15;17)(q22;q21.1 or q12) (Fig 1a–c). The remaining patient with APL (case 580) had a variant form of this translocation involving chromosomes 15, 17, and 7, t(7;17;15)(p22;q21.1 or q12;q22) (Fig 2a and b). The t(15;17) was seen as the sole abnormality in five patients, whereas this rearrangement was accompanied by other karyotypic changes in four others. In the latter group, a gain of chromosome 8 was observed in two cases, a deletion of 6q in one case, and an inversion of chromosome 20 in one case (Table 2).

The patient with CML blastic crisis had a t(15;17) superimposed upon a Ph^1-translocation, t(9;22)(q34;q11), in most marrow cells examined. Among ten cells analyzed at the time of blastic crisis, four showed only a Ph^1, four had a t(15;17) accompanying a Ph^1 (Fig 2c), and two cells showed a missing Y chromosome as well as a t(15;17) and a Ph^1. Chromosome preparations of bone marrow cells at the time of diagnosis were inadequate for analysis. However, pokeweed mitogen-stimulated mononuclear cells from peripheral blood revealed a standard Ph^1 translocation as the sole abnormality in 16 of 24 cells at diagnosis; the eight remain-

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Table 1. Clinical and Hematologic Findings in Nine Patients With APL and One Patient With Promyelocytic Blastic Crisis of CML

Laboratory Case No.*	Age/Sex	Diagnosis	Peripheral Blood			Bone Marrow					Survival† (months)
			WBC (× 10^9/L)	Blasts (%)	Promyelocytes (%)	Blasts (%)	Promyelocytes (%)	DIC	Chemotherapy	Response	
181	32/F	APL	64.6	40	40	42	45	yes	DNR, AC	none	0.1
242	25/F	APL	2.9	20	0	50	35	yes	DNR, AC	CR	19
248	25/M	APL	3.9	35	2	24	53	yes	DNR, AC	none	1.5
282	30/M	APL	1.8	10	3	4	70	yes	DNR, AC	CR	6
303	34/M	APL	20.4	55	10	55	40	yes	DNR, AC	CR	29
547	30/M	APL	1.8	30	35	36	59	yes	DNR, AC	CR	13+
552‡	24/F	APL	2.0	0	0	10	40	yes	DNR, AC	CR	59+
580	35/M	APL	45.4	68	6	61	36	yes	DNR, AC	CR	10+
638	28/M	APL	16.9	10	80	47	45	yes	DNR, AC	CR	6+
545§	38/M	CML blast crisis	32.9	0	1	24	34	yes	AZQ	none	1(26)

Abbreviations: DIC, disseminated intravascular coagulation; DNR, daunorubicin; AC, arabinofuranosil cytosine; CR, complete remission; AZQ, aziridinylbenzoquinone.

*Patients were examined cytogenetically at the time of diagnosis of APL except for those marked with (‡) or (§), in which case the karyotypic study was done in relapse (‡) or at the time of blastic crisis of CML (§). Hematologic data shown here represent findings at the time of the cytogenetic examination.

†Survival is from the time of diagnosis of APL or, in case 545, from the onset of promyelocytic blastic crisis. Number in parentheses in case 545 is the survival from the initial diagnosis of chronic-phase CML. (+), alive as of January 1, 1985. Two patients remain in initial complete remission; patient 547 recently relapsed after a CR of 12 months' duration.

ing cells had a normal karyotype. A detailed description of the clinical course in this patient is reported elsewhere.[9]

The standard or variant t(15;17) was detected in unsynchronized 24-hour cultures from all ten patients examined. In addition, the translocation was observed in methotrexate-synchronized cultures from each of the four patients whose cells were also examined by this modified culture method. On the other hand, metaphase cells showed only normal karyotypes in all three direct specimens examined (cases 181, 242, and 552) (Table 2).

Table 2. Cytogenetic Findings of Nine Patients with APL and One Patient With Promyelocytic Blastic Crisis of CML

Laboratory Case No.*	Sample Source	Karyotype	No. of Metaphase Cells Analyzed†		
			Direct	24-hr	MTX
181	BM	46,XX	9	0	—
		46,XX,t(15;17)(q22;q21.1 or q12)	0	9	—
242	BM	46,XY	13	3	5
		46,XY,t(15;17),del(6)(q21q27?)	0	9	11
248	BM	46,XY	—	2	—
		46,XY,t(15;17)	—	11	—
		46,XY,t(15;17),inv(20)(p13q11 or q13)	—	2	—
282	BM	46,XY	—	3	4
		47,XY,t(15;17),+8	—	2	11
303	PB	46,XY	—	0	6
		46,XY,t(15;17)	—	1	18
547	BM	46,XY	—	4	—
		46,XY,t(15;17)	—	12	—
552‡	BM	46,XX	5	3	—
		47,XX,t(15;17),+8	0	5	—
580	PB	46,XX,t(7;17;15)(p22;q21.1 or q12;q22)	—	6	8
638	PB	46,XY,t(15;17)	—	13	—
545§	BM	46,XY,t(9;22)(q34;q11)	—	4	—
		46,XY,t(15;17),t(9;22)	—	4	—
		45,X,-Y,t(15;17),t(9;22)	—	2	—

Abbreviations: BM, Bone marrow; PB, peripheral blood without mitogen stimulation.

*Patients were examined at the time of diagnosis of APL except for those marked with (‡) and (§), in which case a cytogenetic study was done in relapse of APL (‡) or at the time of blastic crisis of CML (§).

†Number of banded mitoses examined. Direct, direct sample; 24-hour, 24-hour culture without synchronization; MTX, methotrexate-synchronized culture.

DISCUSSION

All nine patients with APL had a standard or variant translocation (15;17). Among approximately 100 acute leukemia patients studied at our center, this rearrangement was

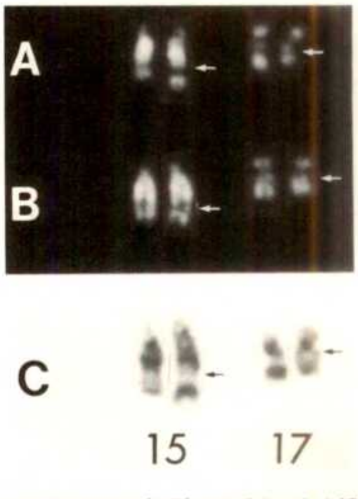

Fig 1. Partial Q-banded (A,B) and G-banded (C) karyotypes showing a 15;17 translocation, t(15;17)(q22;q21.1 or q12), from cases 242(A), 248(B), and 638(C). Arrows indicate possible breakpoints in the defective chromosomes.

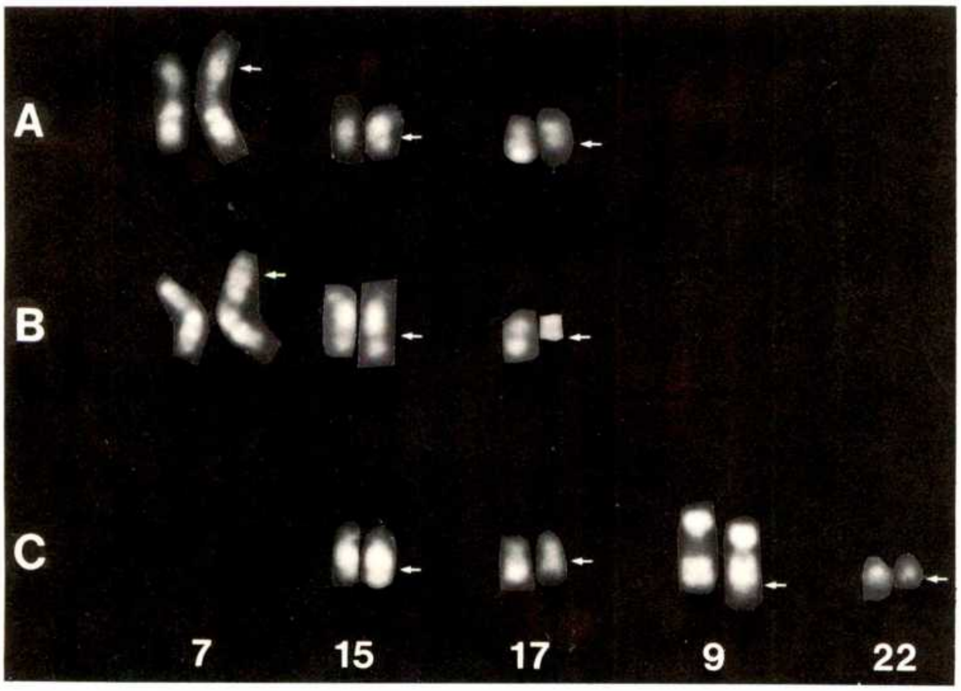

Fig 2. Partial Q-banded karyotypes showing a t(7;17;15)(p22;q21.1 or q12;q22) from case 580(A,B), and a t(15;17) and a t(9;22)(q34;q11) from case 545(C). Arrows indicate proposed breakpoints in the defective chromosomes.

not observed in any other type of acute leukemia. Since the first report of a t(15;17) in APL,[1] this abnormality has consistently been associated with this disease type.[4] The incidence of APL patients with a t(15;17) has differed considerably from one institution to the next, and an unusual geographic distribution of APL cases with the t(15;17) has been noted.[10] For example, the t(15;17) was observed in 27 of 27 patients in Chicago,[5] in 12 of 12 patients in Sapporo, Japan,[6] but in none of 12 patients in Finland and Sweden.[10] It is now recognized that the t(15;17) may be found in a higher percentage of cells in cultured material than in the direct specimen,[4,5,11] and in some cases the t(15;17) could be detected only in cultured samples, as in three of our cases. The reason for the difference in the incidence of chromosome abnormalities between direct and cultured preparations remains to be elucidated. However, Berger et al suggested that mitotic cells in the direct preparations are more likely to be normal erythroblasts, whereas mitotic cells in the cultured specimen are more likely to be leukemic cells since a decrease in normal metaphases in the culture coincides with the disappearance of dividing erythroblasts.[11,12]

Two additional patients with clinical characteristics suggestive of APL, including the presence of DIC, were seen during the same time period of this study. One patient showed light to moderate granulation in some blasts and was classified morphologically as having an M2 leukemia both by light and electron microscopy. Chromosomal banding analysis of 24-hour bone marrow cultures at diagnosis and relapse failed to reveal any karyotypic defects. The other patient was a 27-year-old male with CML blastic crisis; 15% to 20% of circulating and marrow blasts were heavily granulated. Cytogenetic studies revealed a mosaic karyotype: 46,XY, t(9;22)/46,XY,t(8;10),t(9;22)/49,XY,+6,+8p−,+13, t(8;10),t(9;22). Auer's rods could not be identified in either patient. Thus, available evidence suggests that the t(15;17) is highly specific for acute proliferation of leukemic promyelocytes, but DIC and the presence of some cells with increased granulation are not necessarily associated with this translocation.

Four of our cases showed chromosomal changes in addition to the t(15;17). A gain of chromosome 8 was detected in two patients, which is not an uncommon finding in APL.[4,5,13] Furthermore, a +8 is a karyotypic change commonly seen in patients with various types of acute nonlymphocytic leukemia (ANLL), with no specificity for a certain type of ANLL.[4,14]

Even though one of our patients showed a complex three-way translocation, the breakpoints in chromosomes 15 and 17 appeared to be at the same bands involved in the standard t(15;17). Previously, Bernstein et al reported two other cases of complex 15;17 translocations in which chromosomes 15, 17, and either chromosome 2 or 3 were involved.[15] More recently, Ohyashiki et al described a complex four-way translocation (involving chromosomes 1, 5, 15, and 17) in another patient with APL.[16] In all of these cases there is a translocation of the distal part of the long arm of chromosome 17 to the long arm of chromosome 15 and of the distal portion of the long arm of chromosome 15 to a chromosome other than 17. In each case, a characteristic 15q+ abnormality is generated.

Three variant simple translocations have also been described in APL.[17,18] In these cases, the distal portion of 17q was thought to be translocated to either chromosome 1, 7, or 8, without involvement of chromosome 15. However, bone marrow chromosomes often show ill-defined bands in APL, and it is possible that the 15q+ exists in these cases but has been called a chromosome 14.

There has been considerable confusion with regard to the precise location of the breakpoints in the t(15;17) (for review, see ref 19). The marrow chromosomes obtained from patients with APL are often contracted, and this makes difficult exact delineation of the break sites.[20] Hagemeijer et al[21] and Larson et al[5] have examined extended chromosome preparations from patients with the t(15;17), and breakpoints at 15q22 and 17q12 or 17q21.1 were proposed.[5,21] Genetic analysis of the t(15;17) using somatic cell hybridization technology yielded results compatible with this interpretation.[22] The banding in our patients with the standard t(15;17) was also consistent with such a proposal. Moreover, the variant rearrangement seen in one of our patients provided us with the opportunity to examine the defective chromosome 17 without the usual translocation of bands from chromosome 15. In this complex rearrangement 15q22 → 15qter is translocated to chromosome 7 at band 7p22. The derivative 7 retains the medium fluorescent Q band 7p21, and only part of the tiny, weakly fluorescent terminal band (7p22) may be transposed to the derivative 17 chromosome (17q−). The pale fluorescent band retained on the long arm of the 17q− seems to represent all or most of band 17q12. Thus, the banding in the variant translocation appears to provide additional evidence favoring breakpoints at or very near the junction between 17q12 and 17q21 and at 15q22. Dayton et al proposed that the cellular oncogene c-*erb*A may have a role in the development of APL because of its apparent close proximity to the chromosome 17 breakpoint associated with this disease.[23] They assigned c-*erb*A to the q21-22 region of chromosome 17 and provided evidence that the c-*erb*A sequences remain on the 17q− chromosome in APL cells. Therefore, the genetic and karyotypic results, taken together, suggest a breakpoint in the proximal end of band q21(q21.1) of chromosome 17. Rowley has proposed that in APL the 15q+ is the constant recombinant and the critical gene rearrangement leading to malignant transformation is related to the movement of the end of 17q to 15q.[24] Since c-*erb*A apparently is retained on the defective 17 and is not located near the conserved junction on the 15q+, this oncogene may have nothing to do with the disease.

Our patient 545 is the second reported case of a t(15;17) in a promyelocytic form of blastic crisis in CML. The first case was described by Berger et al.[25] Their patient had a chronic phase of 8 months' duration during which time the marrow showed only a Ph^1 translocation. The patient developed promyelocytic blastic crisis with a t(15;17) superimposed upon the Ph^1 cell line. The t(15;17) disappeared from the bone marrow after chemotherapy. The findings in these two cases imply that the translocation (15;17) has an important role in acute malignant proliferation of promyelocytes even in CML cells that already have a specific structural aberration, t(9;22), and are committed to proliferate into granulocytes, erythrocytes, and megakaryocytes.[26] Recently, Moir et al reported a case of acute myelomonocytic leukemia with an increase of eosinophils, basophils, and hypergranular promyelocytes, the latter occasionally showing bundles of Auer rods.[27] Chromosomal studies revealed an inv(16)(p13q22) in 27 of 30 cells. Furthermore, seven of these karyotypically abnormal cells showed a t(15;17) in addition to the inv(16). The appearance of the t(15;17) in association with an increase of abnormal promyelocytes suggested that acute promyelocytic transformation occurred during the course of acute myelomonocytic leukemia. Thus, these observations provide additional evidence for the specificity of certain chromosomal rearrangements for particular target cells The association of a t(15;17) with a specific cell (ie, malignant promyelocytes), even when the clinical diagnosis is different, suggests that the altered chromosomes carry genes important to the function, maturation, and/or proliferation of this cell.

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Treatment of Multiple Myeloma With Recombinant α-Interferon

By Jorge R. Quesada, Raymond Alexanian, Michael Hawkins, Barthel Barlogie, Ernest Borden,
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Thirty-two patients with multiple myeloma were treated with recombinant α-interferon clone A (rIFNαA) daily by intramuscular injection with an initial dose of 12 × 10^6 U/m^2. Of 27 patients evaluable for response, tumor responses were obtained in seven of 14 previously untreated patients (50%) and two of 13 who had relapsed or failed prior chemotherapy (15%). In all patients who had tumor response, there was restoration from subnormal levels of serum immunoglobulins, an effect infrequently observed with chemotherapy. The median duration of tumor responses exceeded 14 months (range, 6 to 20). Moderate-to-severe fatigue was the predominant side effect and necessitated dose reductions in all patients. We conclude that treatment of early stages of multiple myeloma with rIFNαA is beneficial because of the substantial response rate and the improvement in the synthesis of serum immunoglobulins. rIFNαA has a potential role in combination with other agents in the treatment of multiple myeloma.

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EARLY CLINICAL STUDIES of partially purified α-interferon (IFNα) reported antitumor activity in patients with multiple myeloma.[1,2] Subsequent studies have confirmed the activity of both partially purified and cloned IFNα in a limited number of patients.[3–6] In this study we have examined the activity of high doses of recombinant DNA–derived α-interferon (rIFNαA) in patients with multiple myeloma, including both previously untreated patients and those whose disease was refractory to prior chemotherapy.

A high response rate was found among previously untreated patients but limited activity in patients with refractory myeloma. In addition, we present evidence that rIFNαA restored subnormal levels of serum immunoglobulins to normal levels in all responsive patients, an effect not frequently observed in patients responding to chemotherapy.

MATERIALS AND METHODS

Patient population and study design. Thirty-two patients were entered in the study. Criteria for a diagnosis of multiple myeloma included a monoclonal immunoglobulin in serum or monoclonal light chains in urine (or both) and at least one of the following: atypical bone marrow plasmacytosis of 15% or more, osteolytic lesions on x-ray examinations related only to the increased plasma cells, or bone biopsy results revealing plasma cell proliferation Eligibility criteria included a performance status of ≥60 (Karnofsky scale), an expected survival of ≥12 weeks, preserved renal (creatinine level of ≤2.0 mg/mL) and liver function, granulocyte count above 1,500/μL, and a platelet count above 100,000/μL. In all previously treated patients, we required that there were at least 4 weeks between the last treatment and the onset of interferon therapy.

All patients had a clinical examination and staging of their disease before initiation of rIFNαA treatment. Tests performed within 1 week of the first injection included complete blood cell counts, differential count, platelet and reticulocyte counts, 12-channel blood chemistry, coagulation parameters, urinalysis, electrocardiogram, chest x-ray, serum and urine protein electrophoresis, bone marrow examinations, and bone surveys. Serum immunoglobulins were determined by quantitative nephelometry and Bence Jones protein by immunoelectrophoresis. Tumor mass was assessed according to conventional criteria[7,8] All patients were observed by physical examination every 4 to 8 weeks. Peripheral blood cell counts were repeated once or twice each week and blood chemistry, urinalysis, reticulocyte count, and coagulation profile were repeated once a week. Serum and urine electrophoresis were performed every 2 to 4 weeks and bone marrow and bone surveys every 2 to 4 months.

The rIFNαA (Roferon) was provided by Hoffmann LaRoche, Inc (Nutley, NJ). The purified protein was made homogeneous by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, with a specific activity of 2 to 4 × 10^8 U/mg of protein. All patients signed an informed consent to participate in the study according to institutional policies. All patients received an initial dose of 12 × 10^6 U/m^2 by intramuscular injection. Treatment was given daily and continued for a minimum of 12 weeks. A 50% reduction in dose was allowed in the event of evidence of excessive toxicity. Patients who responded continued to receive daily treatment until either tumor progression or the completion of 12 months of treatment

Criteria of response. Tumor response was defined as a ≥50% reduction of serum myeloma protein and disappearance of Bence Jones proteinuria. Response in one patient with nonsecretory multiple myeloma was confirmed when previously marked plasma cell infiltrates disappeared on multiple bone marrow specimens Serum immunoglobulin response was defined as an increase in IgM of ≥40 mg/100 mL to the normal value (≥50 mg/100 mL), an increase in IgA of ≥60 mg/100 mL to the normal value (≥90 mg/100 mL), and an increase in IgG of ≥400 mg/100 mL to the normal value (≥650 mg/100 mL).

RESULTS

Table 1 summarizes the characteristics of the study population, which included 26 patients at the University of Texas M.D. Anderson Hospital and six at Wisconsin Clinical Cancer Center. The extent of tumor mass was low or intermediate in all but four of the previously treated patients. Of the 15 previously untreated patients, eight were asymptomatic, and seven had symptoms referable to lytic lesions.

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Table 1. Patient Characteristics

	Untreated	Prior Chemotherapy
No. of patients	15	17
Median age	57	58
Male/female	11/4	9/8
Protein type		
IgG	10	11
IgA	2	5
Others	3	1
Tumor mass		
High	0	4
Intermediate	5	5
Low	10	8

None of these 15 had hypoalbuminemia, hypercalcemia, or azotemia, but three had a hemoglobin value of <10 g/dL. Thus, most met the criteria for indolent myeloma.[8] All previously treated patients had received combination chemotherapy with intermittent courses of melphalan and prednisone or combinations of alkylating agents and doxorubicin. Four patients were treated upon relapse from prior successful therapy. The remaining patients had disease refractory to preceding combinations of chemotherapy.

Tumor responses. Twenty-seven patients were evaluable for tumor response. Five were not evaluable because they received treatment for less than 2 weeks because of evidence of excessive toxicity. Table 2 shows the results of treatment. Tumor responses were obtained in seven of 14 previously untreated patients (50%), whereas only two of 13 patients responded after failing prior chemotherapy (15%). Among the nine who had responses, seven had a low-tumor mass, and two had an intermediate-tumor mass; five had IgG myeloma protein, three had IgA myeloma protein, and one had a nonsecretory myeloma. Tumor responses occurred within 1 to 4 months (median, 2 months) and lasted from 6 to 20+ months (median, 14+ months). Three previously untreated patients maintained tumor remission beyond 12 months without further interferon therapy. Five of seven previously untreated patients who did not respond to rIFNαA achieved tumor responses with subsequent chemotherapy.

Immunoglobulin response. Restoration of depressed levels of one or two of the normal serum immunoglobulins was observed in all patients who had tumor responses (Fig 1). The time needed to achieve normal immunoglobulin levels in these patients ranged from 3 to 10 months (median, 4.5 months). Restoration was observed in six of six patients with subnormal IgA levels, in seven of nine with with subnormal IgM, and in four of four with subnormal IgG. Figure 2 depicts the pattern of immunoglobulin response in one patient. Concurrent with the reduction of the myeloma protein in this patient, recovery of serum IgA and IgM was first observed after 3 months, with normal levels achieved after 6 months of treatment. In contrast, none of the patients who did not have a tumor response showed a detectable increase in serum immunoglobulins.

Table 2. Tumor Responses of Multiple Myeloma in Recombinant Interferon

	No. Patients (%)	
Results of Treatment	Untreated	Prior Chemotherapy
Tumor response	7 (50)	2 (15)
No change	5 (36)	7 (54)
Progressive disease	2 (14)	4 (31)
Total evaluable patients	14 (100)	13 (100)

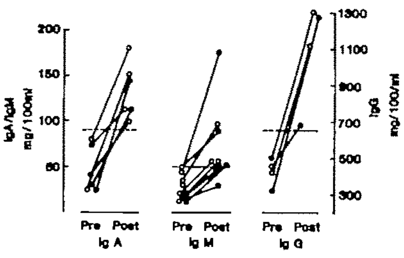

Fig 1. Nonparaprotein serum immunoglobulins in patients with tumor response. Data show pretreatment values and maximum posttreatment serum levels for nine patients with tumor response. Open circles indicate previously untreated patients and closed circles those who had received prior chemotherapy.

Toxicity. All patients were evaluable for toxicity, which was similar to that described in cancer patients receiving daily administration of rIFNαA.[5] Fatigue and asthenia were the most common symptoms and accounted for most treatment interruptions, dose reductions, or drug discontinuations. A 50% dose reduction was indicated within 8 weeks of initiation of treatment in 20 patients (62%) and within 24 weeks in the remaining 12.

Hematologic toxicity included decrements of hemoglobin of 1 to 3 g/dL (median, 1.8 g/dL) within 4 to 12 weeks of treatment in 23 patients (72%). Transient severe granulocytopenia (≤1,500 cells per μL) occurred in five patients (16%). A mild and slow decrease in platelet counts (between 50 and 100,000/μL) was observed in eight patients (25%), in keeping with prior observations.[5] However, rapid onset of thrombocytopenia (within 2 weeks counts below 50,000 cells per μL) consistent with an autoimmune phenomenon but reversible with discontinuation of rIFNαA developed in four other patients.[9] These four patients, as well as one other with high-tumor mass who developed acute tubular necrosis after three doses of rIFNαA, received treatment for less than 2 weeks and were considered to be inevaluable for tumor response. Lastly, mild elevations of serum glutamic oxalo-

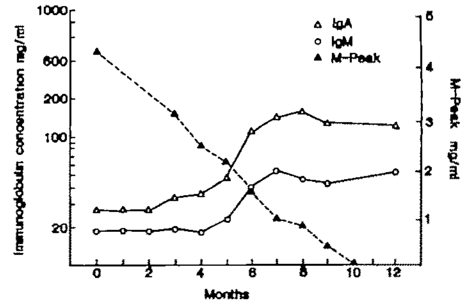

Fig 2. Pattern of recovery of nonparaprotein serum immunoglobulins in a patient with tumor response.

acetic transaminase (42 to 103 U/mL) were found in 16 patients (50%).

DISCUSSION

This study confirmed the therapeutic activity of rIFNαA in patients with multiple myeloma; such activity has been previously noted with partially purified IFNα interferon. We used a highly purified, single-molecular species of IFNα, which has also been effective in inducing remission of other B cell malignancies, including low-grade lymphomas and hairy cell leukemia.[10–13] The 50% response rate among our untreated patients is superior to the 14% response rate recently reported in a study that used partially purified IFNα at lower doses (3 × 10^6 U/d).[4]

The greater activity in our study may be related to the type of interferon, the higher dose, or our selection of patients with early disease. A dose response effect to rIFNαA has been suggested in patients with Kaposi's sarcoma or renal cell carcinoma.[14,15] However, that the present response rate in refractory myeloma is not better than that of other studies and that a higher response rate can be obtained in low–tumor mass myeloma with chemotherapy[16] suggests that patient selection played a predominant role in our results.

Noteworthy is the disparity in the responsiveness among several B cell lymphoid malignancies to IFNα, from the relative resistance of intermediate- and high-grade lymphomas or advanced chronic lymphocytic leukemia,[1,17] to moderate sensitivity of low-grade lymphomas and multiple myeloma,[4–6] and to high susceptibility in hairy cell leukemia.[12] There is at present no information that may relate these different levels of sensitivity with the stage of differentiation or the expression of cellular receptors in the malignant cells. Of interest, however, is the recent finding of plasma cell–associated antigens on hairy cells[18] that provides a phenotypic link between these two IFNα-sensitive tumors.

The recovery of normal levels of serum immunoglobulins in all patients with tumor response contrasts with a similar recovery in only 25% of comparable patients responding to chemotherapy.[19] The deficiency in immunoglobulin synthesis in patients with multiple myeloma has been attributed to macrophage-derived suppressive factors.[20,21] In a murine plasmocytoma model, the inordinate activity of macrophages was induced by a plasma cell–derived activating factor.[22] Consequently, the restoration of normal levels of immunoglobulins in patients with tumor response may be explained by the reduction in tumor mass. However, in contrast to results with chemotherapy, the consistency of the effect in the responsive patients suggests that IFNαA elicited, in addition, favorable cellular interactions between regulatory and immunoglobulin-producing cells. In this regard, both IFNα and highly purified rIFNαA stimulate immunoglobulin production in vitro.[23,24] Such stimulation was dependent on the presence of T helper cells and seemed to be related to the ability of IFNα to enhance the response of B cells to T helper–derived soluble factors.[23]

We conclude that further studies of rIFNαA in patients with early stages of multiple myeloma are justified because of an adequate response rate associated with restoration of synthesis of serum immunoglobulins; further, rIFNαA does not preclude a later tumor response to chemotherapy. In fact, the tolerance to subsequent chemotherapy may be improved by reduction of the risk of infections in patients with a restored humoral immune response. The rIFNαA had acceptable toxicity at an average daily dose of 6 × 10^6 U/m^2. It is unknown whether lower and perhaps less toxic doses might achieve comparable results.

The low response rate in patients previously treated with chemotherapy was similar to our previous observations on rIFNαA in refractory multiple myeloma.[5] However, our findings offer promise for further developments with rIFNαA as part of combination therapies. In this regard, in vitro synergism of IFNα with either γ-interferon, difluoromethylornithine, double-stranded RNA, or some chemotherapeutic agents has already been demonstrated.[25–29] Further, in view of the antitumor activity of high doses of glucocorticoids in refractory myeloma,[30] clinical studies combining steroids and rIFNαA are of interest, both because of potential enhancement of the therapeutic activity of either agent and because of possible improvement in the tolerance to high doses of rIFNαA.

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Defective Interleukin-2 Production and Responsiveness by T Cells in Patients With Chronic Lymphocytic Leukemia of B Cell Variety

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The present studies were designed to investigate the mechanism(s) of the defective T cell proliferative response to various stimuli in patients with B cell chronic lymphocytic leukemia B-CLL. In 14 patients with advanced B-CLL (stage III or IV) we found the T cell response in the autologous (auto) and allogeneic (allo) mixed lymphocyte reaction (MLR) to be 35.7% and 30% of the controls, respectively. Proliferation in the MLR depends upon the production of and response to interleukin 2 (IL 2), a T cell growth factor. IL 2 production in eight B-CLL patients was 22% of the control. The response to IL 2 was measured by the increase in the T cell proliferation in the MLR with the addition of IL 2. T cell proliferation in both the auto and allo MLR of CLL patients was significantly lower than in the controls after the addition of IL 2. The proliferative response of normal T cells to stimulation by CLL B cells was 50% of the control. This latter response was increased to control levels when cultures were supplemented with exogenous IL 2, suggesting that CLL B cells could stimulate IL 2 receptor generation in normal T cells in an allo MLR, but not IL 2 production. The presence of IL 2 receptors on activated T cells was directly determined using anti-Tac, a monoclonal antibody with specificity for the IL 2 receptor. Of the mitogen- or MLR-activated T cells in CLL patients, 6% and 10%, respectively, expressed Tac antigen, whereas identically stimulated control T cells were 60% and 47% Tac+, respectively. Our findings suggest that T cells in B-CLL are defective in their recognition of self or foreign major histocompatibility antigens as demonstrated by their impaired responsiveness in the MLR. Thus, these cells are unable to produce IL 2 or generate IL 2 receptors.

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B CELL chronic lymphocytic leukemia (B-CLL) is a malignancy in which immature, immunoincompetent B cells progressively accumulate in the bone marrow, peripheral blood, and other organs of the reticuloendothelial system.[1-3]

Recently, T cells in B-CLL were shown to be abnormal, demonstrating a diminished proliferative response to T cell mitogens, as well as to autologous (auto) or allogeneic (allo) cells in the mixed lymphocyte reaction (MLR).[4-7] Effector and regulatory T cells, ie, cytotoxic, helper, inducer, or suppressor cells, are generated during the MLR.[8,9] Aberrant T cell responses in the auto and allo MLR could reflect a defect in the immunoregulatory T cell generation in B-CLL patients.

Mitogen- or MLR-activated T cells produce a T cell growth factor, IL 2, and generate its receptors on the cell surface.[10,11] After IL 2 binds to its receptor, the T cells proliferate and differentiate into regulatory or effector cells with helper, suppressor, or cytotoxic functions. Abnormalities in IL 2 production and responsiveness by T cells have been implicated in the pathogenesis of autoimmune diseases such as systemic lupus erythematosus, the acquired immunodeficiency syndrome (AIDS), and in acute lymphocytic leukemia.[12,13] In the present study, T lymphocytes from 14 patients with advanced B-CLL were found to be defective in IL 2 production and receptor generation upon stimulation with mitogen or antigen. We suggest that these defects could account for the T cell abnormalities noted in B-CLL.

MATERIAL AND METHODS

Patients. A total of 14 untreated patients with B-CLL were studied (Table 1). The diagnosis was made by physical examination and by observation of patients' Wright-Giemsa–stained peripheral blood and bone marrow smears. All patients studied had B-CLL as determined by surface immunoglobulin (SIg) evaluation of their peripheral blood lymphocytes (PBL).[14] All patients were staged according to the Rai classification and had stage III or IV disease.[15] Controls were healthy age-matched volunteers.

Isolation of human lymphocytes and subpopulations. PBL from patients and controls were separated from heparinized whole blood (50 mL) by Ficoll-Hypaque centrifugation (FHC) (lymphocyte separation medium, Bionetics laboratory products, Litton Bionetics, Kensington Md).[16] Unfractionated mononuclear cells were depleted of macrophages by adherence to plastic and adherent and nonadherent cells collected. Macrophage-depleted mononuclear cells were then separated into E rosette–positive (E+) and E rosette–negative (E−) populations with neuraminidase (Sigma Chemical Co, St Louis) treated 5% sheep red blood cells (SRBC) (GIBCO Laboratories, Madison, Wis) E+ cells were enumerated and recovered as described.[17] Briefly, macrophage-depleted PBL (4×10^6/mL) in 2 mL of RPMI 1640 (GIBCO Laboratories, Chagrin Falls, Ohio) were mixed with 2 ml of 1% neuraminidase (25 U/mL of 5% SRBC) treated SRBC and 0.5 mL of fetal calf serum (GIBCO Laboratories, Chagrin Falls, Ohio) (inactivated at 56 °C for one hour) The mixture was incubated at 37 °C for five minutes, centrifuged at 200 g for five minutes, and incubated on ice (4 °C) for four hours. The rosettes were carefully resuspended by rotation of the tubes, incubated for an additional 15 minutes on ice, and subjected to FHC. E+ cells were recovered from the pellet after lysis of attached SRBC by treatment with tris(hydroxymethyl)aminomethane-buffered 0.83% ammonium chloride (pH 7 2) for five minutes at 37 °C, and the T cells were washed three times with RPMI 1640 and rerosetted with SRBC. The E+ cells separated after double rosetting of PBL contained <1% SIg+, <3% alpha-naphthyl-acetate-esterase–positive cells, and >97% formed rosettes with SRBC. Of the E+ cells, 95% ± 1% reacted with a monoclonal antibody, anti-T11 (Ortho Diagnostics, Westwood, Mass), that defines the SRBC receptor present on all mature peripheral lymphocytes.[18] Unrosetted cells at the Ficoll-medium interface are referred

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Table 1. Hematologic Data in 14 Patients With B-CLL

Patients	Hemoglobin (mg/100 mL)	Lymphocytes 10^3/mL	E+ (%)	SIg+, E− (%)	SIg+ Cells' Light-Chain Isotype	Platelets/mm^3
1	9	67	30	67	λ	50,000
2	9	25	30	60	λ	100,000
3	10	30	28	65	λ	120,000
4	6	110	6	94	κ	78,000
5	12	150	3	97	λ	15,000
6	13	170	5	90	κ	95,000
7	10	89	25	60	κ	50,000
8	12	50	7	90	κ	63,000
9	7	20	10	80	λ	100,000
10	5	170	5	90	λ	42,000
11	6	140	6	94	κ	22,000
12	5	200	3	90	κ	45,000
13	9	59	20	75	κ	94,000
14	11	16	12	80	κ	91,000

to as B cells and contained 96% SIg+ cells, <1% E+ cells, and <3% alpha-naphtyl-acetate-esterase–positive cells. In the case of CLL, B cells were typed for IgM, IgD, IgG, κ and λ light chains using fluorescein isothyocyanate (FITC)-labeled rabbit antihuman immunoglobulin (RaHIg) specific antiserum (Cappel Laboratories, Cochranville, Pa). B cells were also counted by rosetting with SRBC coated with IgM antibody and complement. Cell viability after separation was determined by exclusion of 0.05% (vol:vol) trypan blue in phosphate-buffered saline and was >95%. When subjected to immunofluorescence with FITC-conjugated rabbit antihuman κ or λ antiserum, the CLL B cells stain lightly compared to normal B cells, and 98% of these cells were positive for either κ or λ light-chain expression.

Adherent cells were recovered from plastic Petri dishes and quantitated. These cells were 98% alpha-naphtyl-esterase–positive and >95% reacted with a monoclonal antibody, M5 (Coulter Immunology, Hialeah, Fla), that identifies peripheral blood monocytes and macrophages.[19]

The auto and allo MLR. For the allo MLR, responder T cells and stimulator B cells were obtained sterilely from unrelated individuals. In the auto MLR, responder T cells and the stimulator B cells were obtained from a single individual's blood sample. B cells (10 to 30 x 10^6) to be used as stimulator cells were suspended in 1.5 mL of RPMI 1640, treated with 66 μg/mL of mitomycin C (Sigma) for 30 minutes at 37 °C, and then washed three times. Cells to be used in culture were resuspended to a final concentration of 2 x 10^6/mL in RPMI, supplemented with 10% heat-inactivated pooled human AB serum, 10 mmol/L HEPES (GIBCO, Grand Island, NY), 2mmol/L L-glutamine (GIBCO, Grand Island, NY), and antibiotics (complete medium). Responder and stimulator cells (0.1 mL of each) were cocultured in flat-bottomed Microtest tissue culture plates (No. 3040, Falcon Labware, Oxnard, Calif) for six days at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Eighteen hours before harvesting the cultures, 1 μCi of tritiated thymidine (2 Ci/mmol, Schwarz/Mann, Spring Valley, NY) was added to each well. Twenty-four hours later the samples were harvested with a microculture harvesting device, placed in 5 mL of Scintiverse (Fisher Scientific Co, Fair Lawn, NJ), and counted in a liquid scintillation spectrophotometer. All cultures were performed in triplicate with controls consisting of T cells by themselves and mitomycin C–treated stimulator cells by themselves. All data were calculated as the difference between counts per minute (cpm) of cultures containing stimulator and responder cells and cpm of cultures containing responder cells alone plus the cpm of stimulator cells alone. The lymphocytes from each patient were compared to two controls simultaneously isolated and cultured.

IL 2 production and assay. Monocyte-depleted T cells (5 x 10^5/mL) were incubated with 1 μg/mL of phytohemagglutinin (PHA-P) (Burroughs Wellcome Co, Greenville, NC) and 4 ng/mL of 4B phorbol 12-myristate 13-acetate (PMA) (Sigma) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air for 48 hours in complete medium supplemented with 2% human AB serum in 12 x 75-mm culture tubes (Fisher Scientific Co, Pittsburgh, Pa).[20] Cell viability after culture was greater than 95%. The supernatant was collected and filtered through a 0.45-μ filter (Nalgene Labware, Nalge Co, Rochester, NY) before storage at −70 °C. Preliminary experiments demonstrated this cell concentration and dosage of mitogen to be optimal for IL 2 production. The IL 2 concentration in the supernatants was measured using a cytotoxic T lymphocyte (CTLL) assay as previously described.[21] The units of IL 2 were determined by probit analysis. The IL 2 standard, a 1:16 dilution of rat IL 2 that was the supernatant obtained from spleen cells (10 x 10^6/mL) incubated with 5 μg/mL of Concanavalin A for 48 hours, was denoted as 1 U/mL.

Response to IL 2. The auto MLR was cultured in medium alone or medium supplemented 1.4 (vol.vol) with various concentrations of IL 2 (Electro Nucleonics, Inc, Silver Spring, Md). This IL 2 preparation contained less than 20 pg/mL PHA as determined by an indirect, enzyme-linked, immunoabsorbent assay. This amount of PHA did not cause an increase in T cell proliferation when added to the auto MLR. To some cultures purified IL 1 (Genzyme, Boston, 100 U/mL) was added (1.80, vol:vol).

Immunofluorescence with anti-Tac. Patient or control T cells (2 x 10^6/mL) activated for two days with PHA-P (1 μg/mL) and PMA (4 ng/mL) or for six days with auto or allo stimulator cells (2 x 10^6/mL) were tested for reactivity with anti-Tac (a generous gift from Dr T.A. Waldman) antibody (1:500) by indirect immunofluorescence using FITC-labeled goat antimouse IgG antibody (GAMIgG). Immunofluorescent cells were examined using a fluorescent microscope (Ortholux II, Leitz, Wetzlau, Germany) as described.[22]

Statistical evaluation. Student's *t* test was used to determine the significance of the difference between experimental results obtained from patients and controls.

RESULTS

To determine the nature of T cell defects in patients with B-CLL we first investigated the proliferative responses of their T cells to auto and allo B cells in a MLR. In the patient population the mean T cell proliferation in an auto MLR was significantly lower than control values, 1,800 ± 582 cpm *v*

5,033 ± 1,200 cpm, respectively ($P < .01$) (Fig 1). Since B cells in CLL are monoclonal, displaying either κ or λ light chains, it is possible that they have a limited display of class II major histocompatibility locus (MHC) antigens and thus would be defective as stimulator cells in the auto MLR. Therefore, normal B cells were used to stimulate CLL T cells to determine whether these cells responded appropriately in an allo MLR. T cells from CLL patients had a defective response to allo control B cells compared to control values 6,000 ± 750 cpm *v* 22,582 ± 2,200 cpm respectively ($P < .001$) (Fig 2). These findings suggested that T cells were defective as responders in both auto and allo MLR in B-CLL patients.

To determine whether there was, in addition to a responder T cell defect, an impairment in the CLL B cells to act as stimulators, an allo MLR was performed using normal T cells as responders and CLL B cells as stimulators. In these allo MLR, normal T cells were stimulated by either CLL B cells or allo B cells from healthy controls. As seen in Fig 2, the CLL B cell induced a T cell proliferation that was 50% of that induced by control B cells, ie, 10,231 ± 2,310 cpm *v* 22,582 ± 2,200 cpm respectively ($P < .05$). These results indicate that defects in both stimulatory B cells as well as responder T cells are responsible for the low auto and allo MLR in patients with B-CLL.

During auto and allo MLR, T cells proliferate in response to class II MHC antigens present on the surface of non-T lymphocytes and as a consequence of this activation T cells produce IL 2 and generate IL 2 receptors.[11,21] Since the binding of IL 2 to its receptor induces the proliferation and clonal expansion of the activated T cells, we measured the IL 2 production and receptor generation in T cells from B-CLL patients. In eight of our patients we determined the capacity of their T cells to produce IL 2 upon activation with PHA-P and PMA rather than auto MLR activation since the amount of IL 2 generated in control and patient auto MLR was below the sensitivity of the CTLL assay. After lectin stimulation, patients' T cells produced 22% of the IL 2 produced by control lymphocytes ($P < .01$) (Fig 3). The presence of IL 2 receptor on activated T cells was determined using a monoclonal antibody, anti-Tac, with specificity for the IL 2 receptor.[23] When T cells from CLL patients were activated with PHA-P and PMA, only 6% ± 2% of them developed Tac+ compared to control T cells that demonstrated 60% ± 10% Tac+ cells (Table 2). When CLL and control T cells were activated in an auto MLR there were 7% ± 2% Tac+ cells *v* 25% ± 5% Tac+ cells, respectively. Similarly, T cells from CLL patients and controls were activated in an allo MLR and Tac positivity determined followed culture. After such activation there were 10% ± 5% Tac+ cells in CLL patients compared to 47% ± 5% in controls. Finally, we found the same percentage of Tac+ control T cells (47%) regardless of their activation with allo control B cells or CLL B cells in an allo MLR.

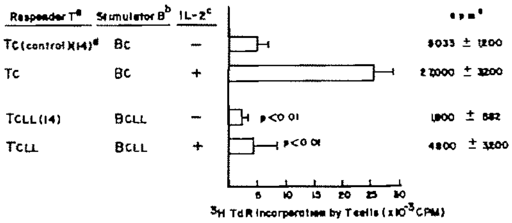

Fig 1. Autologous MLR was performed as described by incubating 2 × 10^5 responder T cells (a) with 2 × 10^5 mitomycin C–treated auto B cells (b) for six days at 37 °C and 5% CO_2/95% air in a humidified atmosphere. To some of these cultures purified human IL 2 was added (1:4 vol:vol) (c) The number of individuals studied is (d), and the cpm of responder T cells is (e).

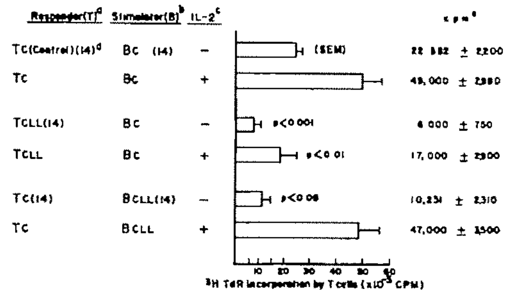

Fig 2. Allogeneic MLR was performed as described by incubating 2 × 10^5 responder T cells (a) with 2 × 10^5 mitomycin C–treated allo control B cells (c) or leukemic B cells (P) for six days at 37 °C and 5% CO_2/95% air in a humidified atmosphere. To some of these cultures purified human IL 2 was added (1:4 vol:vol) (c) The number of individuals studied is (d), and the cpm of responder T cells is (e).

These results demonstrate that CLL B cells are able to stimulate generation of IL 2 receptors but not IL 2 production in normal T cells. To verify these findings we added IL 2 to T and B cells cultured for MLR. The addition of IL 2 to allo MLR cultures containing normal T cells and CLL B cells corrected the low proliferative response, ie, without IL 2, 10,231 cpm, and with IL 2, 47,000 cpm, compared to that of normal allo MLR cultured with IL 2, 49,000 ± 2,990 cpm (Fig 2). These results substantiate the results of previous immunofluorescence experiments performed with anti-Tac that indicate that CLL B cells can induce IL 2 receptor generation but not IL 2 production from normal T cells.

Further studies were directed at determining the nature of the T cell defect in CLL patients. Auto and allo MLR were performed using T cells from CLL patients as responder and normal and CLL B cells as stimulators along with IL 2 in the culture media. In the auto MLR of CLL patients, the T cell proliferation increased only from 1,800 cpm to 4,800 cpm with the addition of IL 2, whereas there was a significant rise from 5,033 cpm to 27,000 cpm in the control auto MLR ($P < .001$) (Fig 1). In the allo MLR with CLL T cells as responders and normal B cells as stimulators, the proliferation increased from 6,000 cpm to 17,000 cpm with the addition of IL 2 (Fig 2). This increase was substantially less than the proliferative increase one sees with control T and B cells in allo MLR upon the addition of IL 2, ie, 49,000 cpm ($P < .05$). These results, presented in Table 2, are consistent with the results of immunofluorescence experiments using anti-Tac antibody and demonstrate that not only is Tac expansion significantly diminished in T cells activated in an allo MLR but there are no other functional IL 2 receptors on these cells in B-CLL.

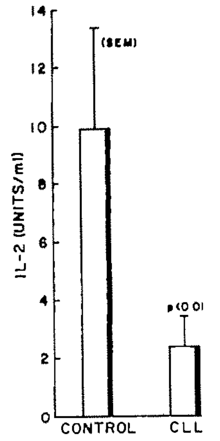

Fig 3. IL 2 production was quantitated in supernatants obtained from monocyte-depleted T cells (5×10^6/mL) stimulated with PHA-P (1 μg/mL) and PMA (4 ng/mL) for 48 hours at 37 °C and 5% CO_2/95% air in a humidified atmosphere. The IL 2 concentration in the supernatants was measured using a CTLL assay. The number of patients and controls who were studied for IL 2 production is 12.

DISCUSSION

These results demonstrate that T cells as well as B cells of patients with B-CLL have intrinsic defects that result in ineffective cellular interactions required for certain immune responses. The T cells of patients with advanced CLL are unable to produce IL 2 or generate IL 2 receptors following activation with mitogen, auto cells, or allo cells. The B cells of these patients are unable to induce normal T cells to produce IL 2, although they are capable of inducing IL 2 receptors on normal T lymphocytes. These experiments do not allow us to determine which step of T cell activation prior to IL 2 receptor generation is defective in B-CLL. The release of IL 2 by T cells occurs in response to two signals provided by antigen-stimulated macrophages, ie, presentation of antigen and production of interleukin 1 (IL 1), a mediator that binds to activated T cells and stimulates the production of IL 2.[24] By performing our experiments using adherent cell-depleted T cells, we were able to provide similar numbers of macrophages, <3%, for patients and controls. In experiments measuring IL 2 synthesis, we added PMA to cultures since it enables lymphocytes to produce IL 2 in response to mitogens in the absence of adherent cells.[25] The quantity of IL 2 in the supernatant of PHA-P–activated T cells was similar regardless of the presence or absence of PMA, suggesting that defective IL 1 production alone could not explain the inability of T cells to produce IL 2 in B-CLL. Furthermore, the addition of purified IL 1 to MLR cultures did not change the proliferation of CLL T cells (not shown).

Table 2. Defective T Cell Activation in B-CLL Patients. Percentage of Tac+ Cells Following Activation of T Cells

Source of T Cells	Mitogen*	Auto B Cells†	Allo B Cells‡	CLL B Cells§
Controls (6)[‖]	60 ± 10	25 ± 5	47 ± 5	47 ± 10
B-CLL (6)	6 ± 2	7 ± 2	10 ± 5	ND

Abbreviation: ND, not determined.

*T cells (1×10^6/mL) were stimulated with PHA-P (1 μg/mL) and PMA (4 ng/mL) for three days

†T cells (1×10^6/mL) were stimulated with mitomycin C–treated auto B cells (1×10^6/mL) for six days.

‡T cells (1×10^6/mL) were stimulated with mitomycin C–treated allo B cells (1×10^6/mL) for six days

§Control T cells (1×10^6/mL) were stimulated with mitomycin C–treated CLL B cells for six days.

[‖] Number of persons studied.

Tac+ cells were determined using monoclonal anti-Tac antibody and indirect immunofluorescence.

In all patients, there was a significantly diminished T cell proliferation in response to auto and allo cells compared to control T cells. In the auto MLR, auto T cells recognize the class II MHC antigens (DR antigens) on the surface of non-T cells and respond by proliferating.[26,27] These activated T lymphocytes may then function as immunoregulatory cells with the capacity to augment or suppress immune responses. Recognition of class II MHC antigens on the antigen-presenting cell is particularly important since the T cell that proliferates upon exposure to exogenous antigens such as virus recognizes virus in association with self-histocompatibility antigen (DR/Ia) on the antigen-presenting cell.[28,29] Lack of DR/Ia recognition could result in an inability to generate cytotoxic cells that could result in fulminant viral infections as encountered in patients with CLL. In our patients the low auto MLR did not increase to normal levels with exogenous IL 2 addition, suggesting a lack of IL 2 receptor generation in T cells stimulated with auto cells. This finding was substantiated by demonstrating the inability to induce Tac antigen expression, an IL 2 receptor, following T cell activation with auto stimulated cells. The diminished proliferation and expression of Tac antigen following auto stimulation could be due to a defect in the responder cell, the stimulator cell, or both. Therefore, we measured the response of T cells from B-CLL patients to normal B cells in an allo MLR. T cells from B-CLL patients had a substantially decreased proliferative response in the allo MLR. Proliferation in the allo MLR is also dependent upon IL 2 production and receptor generation.[30,31] Exogenous IL 2 partially corrected the defective allo MLR, although it was still significantly less than controls. Although a suppressive effect of leukemic B cells on T cell functions cannot be ruled out, our results suggest that T cells have an intrinsic defect in B-CLL since they did not respond to either auto or allo cellular antigens by IL 2 production and receptor generation as normal T cells did. An alternative explanation of our findings could be the presence of a low helper (T4+)/suppressor (T8+) cell ratio in the peripheral blood in these patients.[32] This is unlikely since in four of the presented patients isolated T4+ as well as T8+ cells had a diminished proliferative response to T cell mitogens and to allo cells. In addition, production of IL 2 was significantly decreased in both of the stimulated T cell subsets (not shown).

The capacity of B cells from CLL patients to stimulate a proliferative response in normal T cells (allo MLR) was noted to be significantly diminished. This stimulatory defect was corrected by exogenous IL 2. Further studies demonstrated that the CLL B cells were able to induce IL 2 receptors on normal T cells, although they lacked the capacity to induce IL 2 synthesis in normal T cells. Thus the addition of IL 2 would be expected to normalize the allo MLR using CLL B cells to stimulate normal T cells. Although all of the patients studied expressed DR antigen on their leukemic B cells (not shown), as determined with immunofluorescence using a monoclonal anti-Ia antibody (antinonpolymorphic HLA-DR), it is possible that the leukemic B cells lack the MHC class II determinant that is required to induce IL 2 production by T cells although they express another determinant required for IL 2 receptor generation. It has been shown that leukemic B cells in certain patients with B-CLL and hairy cell leukemia do not express a DR-linked determinant, DQ, although they react with MAb against monomorphic DR determinants.[33,34] The relationship between DQ and/or DR expression by B cells and their capacity to induce IL 2 production and/or receptor generation among T cells is currently being studied.

These results raise two important questions: why are T cells intrinsically defective in B-CLL and what role do the T cell defects play in the pathogenesis of this disease? Initial studies using G6PD expression or cyogenetic analysis as clonal markers of hematopoietic cells have shown that T cells and leukemic B cells arise from different clones of hematopoietic stem cells in a few patients with B-CLL.[35] A larger population of patients will have to be analyzed using chromosome banding to determine whether leukemic B cells and T cells do indeed arise from the same clone of a leukemic lymphoid stem cell. It is also possible that T cells are transformed by a virus that changes their immunoregulatory effect on B cells or that both T and B cells are transformed. Human T cell leukemia virus, HTLV-1, a retrovirus, has recently been found to be responsible for inducing a T cell leukemia/lymphoma in certain areas of the world.[36] Proviral sequences of this retrovirus were shown to be present in T cells from 35 B-CLL cases diagnosed in Jamaica.[37] Effector and regulatory T cell defects in B-CLL could be of pathogenic significance.

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Immunohistochemical Localization of Membrane and α-Granule Proteins in Human Megakaryocytes: Application to Plastic-Embedded Bone Marrow Biopsy Specimens

By Jay H. Beckstead, Paula E. Stenberg, Rodger P. McEver, Marc A. Shuman, and Dorothy F. Bainton

Using a new technique for antigen localization, we have demonstrated platelet proteins in megakaryocytes in plastic-embedded biopsy specimens of normal human bone marrow. In a series of 25 specimens, megakaryocytes showed labeling with antibodies to the integral membrane glycoproteins IIIa, IIb, and the IIb-IIIa complex; granule membrane protein 140; and five alpha-granule matrix proteins: thrombospondin, factor VIII-related antigen, β-thromboglobulin, platelet factor 4, and fibrinogen. The antibodies to the membrane glycoproteins IIIa, IIb, and IIb-IIIa produced diffuse cytoplasmic staining and heavier staining on the plasma membrane, whereas the antibodies to the α-granule matrix proteins produced a distinct granular staining within the cytoplasm. Staining for granule membrane protein 140 was also granular in distribution. Rare mononuclear cells consistent with megakaryocyte precursors were labeled with these markers. Other enzyme histochemical and lectin-binding studies showed that the enzyme α-naphthyl acetate esterase, the lectin Ulex europaeus I, and the periodic-acid Schiff reaction were consistent, but not specific, markers of megakaryocytes. This immunohistochemical technique should facilitate the examination of qualitative and quantitative changes in megakaryocytes in a variety of physiologic and pathologic processes.

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PLATELETS, anucleate blood cells that play a critical role in hemostasis, are derived from megakaryocytes. Since platelets have little capacity for protein synthesis, most of the proteins essential for their function are thought to be produced and packaged by the megakaryocyte.

Although a number of recent studies have demonstrated platelet proteins to be present in megakaryocytes,[1-3] the techniques used to localize these substances have been cumbersome or have not provided optimal morphologic correlation. An additional problem has been the difficulty of demonstrating these antigens in intact specimens of bone marrow. The ability to do so could improve our understanding of the basic biology and pathology of megakaryocytes and platelets. It has become apparent that the megakaryocyte and its precursors may be involved in a wide range of pathologic processes, including acute megakaryoblastic leukemia, blast transformation of chronic myelogenous leukemia, acute myelofibrosis, myelodysplastic syndromes, and myeloproliferative disorders.[4-10] The ability to localize specific megakaryocyte antigens in well-preserved specimens of human bone marrow could significantly contribute to our understanding of these and other disorders.

The plastic-embedding procedure developed in our laboratory[11] was originally designed for maximal preservation of diagnostically useful enzymes. We subsequently noted that a very limited number of antigens could be detected in the same sections using an immunohistochemical technique.[12] We have further modified the fixation, embedding procedure, and immunohistochemical technique to enable us to accurately localize a broad spectrum of antigens characteristic of human platelets and megakaryocytes. In the present study, we have used this technique to demonstrate five α-granule constituents—factor VIII–related antigen (VIIIR:Ag), thrombospondin, fibrinogen, β-thromboglobulin (βTG), and platelet factor 4 (PF4)—three platelet membrane glycoproteins (GPIIIa, GPIIb, and the GPIIb-IIIa complex), and a granule membrane protein (GMP140) in megakaryocytes in sections of normal human bone marrow. We have also performed several enzyme histochemical procedures and lectin-binding reactions on the same sections. We believe that this method provides the best antigen localization currently available in biopsy specimens.

MATERIALS AND METHODS

A series of 25 specimens of human bone marrow was obtained after informed consent was given All were from patients with normal platelet counts who were undergoing evaluation for malignancies or mild anemias and whose biopsy specimens were morphologically normal. The specimens were obtained by Jamshidi needle biopsy of the posterior iliac crest They were fixed immediately at 4 °C in 1% paraformaldehyde in 0.1 mol/L PO_4 buffer, pH 7.4 and were kept in this fixative for one to 12 hours at 4 °C. They were further processed in an Autotechnicon Tissue Processor (Technicon, Tarrytown, NY) in a cold room (4 °C) as follows. (1) fixed for six hours in 4% paraformaldehyde in 0.1 mol/L PO_4 buffer; (2) washed in 0.1 mol/L PO_4 buffer with 2% sucrose and 50 mmol/L NH_4Cl for one hour, repeated twice; (3) washed in 0.1 mol/L PO_4 buffer with 50 mmol/L NH_4Cl for one hour, repeated once; (4) dehydrated in 50% acetone for one hour, 100% acetone for two hours, 50 parts acetone/50 parts glycol methacrylate monomer for one hour; (5) infiltrated with 100% glycol methacrylate monomer for one hour; (6) changed to 100% glycol methacrylate monomer and infiltrated for approximately four hours. The specimens were then embedded in a mixture composed of glycol methacrylate monomer (20 mL), benzol peroxide (0.09 g), and polyethylene glycol 400 with *N,N*-dimethylaniline (0.5 mL) (Polysciences Inc, Warrington, Pa) The embedded tissue was placed under vacuum (15 to 20 mm Hg) at 4 °C and allowed to polymerize overnight. Sections were then cut at 1 to 3 μm with glass knives on a Sorvall JB-4 microtome (Dupont, Wilmington, Del). The sections were transferred via water to coverslips and air dried at room temperature.

Routine staining procedures and enzyme histochemistry were performed as previously described.[11-13] These included hematoxylin-

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eosin-azure stain, the periodic-acid Schiff (PAS) reaction with and without digestase, enzyme histochemical reactions for α-naphthyl acetate esterase and α-naphthyl butyrate esterase with and without sodium fluoride, and acid phosphatase with and without tartaric acid, alkaline phosphatase, adenosine triphosphatase (ATPase), and 5′-nucleotidase The immunohistochemical procedure is a modification of a biotin-avidin procedure,[14] whereas the lectin used was directly conjugated to peroxidase.

Immunohistochemical procedures on plastic sections were performed in the following sequence. Unless otherwise specified, all incubations were carried out at room temperature.

1. Digestion with 0.25% trypsin[15] in Saline A (GIBCO, Santa Clara, Calif), pH 7.6, for ten minutes at 37 °C.

2. Incubation with 3% normal serum (same species as secondary antibody) in Ca^{++}- and Mg^{++}-free phosphate-buffered saline (CMF-PBS) for 30 minutes at 37 °C.

3. Incubation overnight at 4 °C with one of the following antisera. All dilutions were made in CMF-PBS with 3% normal goat or horse serum (same species as secondary antibody), and sections were rinsed between incubations with CMF-PBS. (*a*) Polyclonal antibodies included rabbit antihuman VIIIR:Ag (Dako, Santa Barbara, Calif, 1:1,000); rabbit antihuman βTG antiserum[16] (1 mg/mL 1:2,000); rabbit antihuman fibrinogen antiserum (affinity purified by Dr David Phillips, Gladstone Foundation, San Francisco,[16] 1:5,000); rabbit antihuman PF4 antiserum (affinity purified by Dr Shirley P. Levine,[17] 1.2,000); rabbit antihuman platelet membrane GPIIIa[18] (1.5,000); rabbit antihuman platelet membrane GPIIb (1:5,000). (b) Monoclonal antibodies included mouse antihuman HLA-DR/Ia (0.25 mg/mL) (Coulter Immunology, Hialeah, Fla, 1.500); mouse antihuman VIIIR:Ag (4.32 mg/mL) (Dako, 1:100); mouse antihuman thrombospondin (produced by M.J. Doyle and D. Mosher, University of Wisconsin, School of Medicine, 1:5,000); mouse antihuman platelet GMP140 (7.5 mg/mL) (S-12,[19] 1:500); mouse antihuman platelet membrane GPIIb (3 9 mg/mL) (Tab,[20] 1·500); mouse antihuman platelet membrane GPIIb-IIIa complex (2.0 mg/mL) (T-10,[20] 1:500); and mouse antihuman platelet membrane GPIIIa (0.232 mg/mL) (produced by P J. Newman, Blood Center of Southeastern Wisconsin, AP-3[21], 1:50).

4. Incubation with the appropriate secondary antibody, biotinylated goat antirabbit IgG (Vector, Burlingame, Calif, 1:400) or biotinylated horse antimouse IgG (Vector, 1·200) for one hour.

5. Inhibition of endogenous peroxidase by incubation with 0.3% H_2O_2 in 30% methanol[22] for three minutes.

6. Incubation with the avidin-biotin complex (Vector; 1:80) or Strepavidin-peroxidase (BioGenex, Dublin, Calif, undiluted) for one hour.

7. Development of peroxidase reaction by preincubation with 0.05% 3,3′-diaminobenzidine in CMF-PBS for ten minutes, followed by incubation with a CMF-PBS solution containing 0.05% diaminobenzidine, 0.1% H_2O_2,[23] 0 1 mol/L imidazole,[24] and 0.1 mol/L sodium azide[25] for five minutes; the reaction is further enhanced by incubation with a solution of 0.5% $CuSO_4$[26] for five minutes.

8. Counterstaining with Gill's hematoxylin 2 for 1.5 minutes, blueing with Scott's water (0.2% Na bicarbonate and 1% $MgSo_4$ in tap H_2O), and drying and mounting with Permount (Fisher Scientific, Fairlawn, NJ).

Lectin histochemical procedures on plastic sections were performed in the following sequence (unless otherwise specified, all incubations were carried out at room temperature): (1) digestion with 0.25% trypsin in Saline A for ten minutes at 37 °C; (2) incubation with 1% ovalbumin (Sigma Chemical Co, St Louis) in CMF-PBS for 30 minutes at 37 °C; (3) inhibition of endogenous peroxidase as previously stated, (4) incubation with Ulex europaeus I lectin directly conjugated to peroxidase (E-Y, San Mateo, Calif, 1:50) for two hours; and (5) development of peroxidase reaction and counterstaining as previously mentioned.

Control procedures included the substitution of irrelevant antibodies, preimmune serum, or ascites fluid for the primary antibodies and the elimination of primary or secondary reagents.

All biopsy specimens were examined using a Zeiss microscope and a 40× objective. All morphologically identifiable megakaryocytes were counted in each specimen with each antibody, (range, 32 to 174; mean, 87). Morphologically identifiable megakaryocytes were defined as cells with large nuclei (greater than 1.5 times the diameter of a myeloblast) and moderate-to-low nuclear/cytoplasmic (N/C) ratios. Those with low N/C ratios and granular cytoplasm were considered mature megakaryocytes; those with moderate N/C ratios were considered immature megakaryocytes. In addition, rare mononuclear cells the size of myeloblasts or smaller with high N/C ratios were assumed to be megakaryocyte precursors if they showed positive reactions with the antibody reagents. Although these cells are not morphologically identifiable, control preparations showing an absence of staining in myeloid, erythroid, monocytic, or lymphoid precursors make this a reasonable assumption.

Cells for electron microscopy were washed from marrow specimens into 2% paraformaldehyde–0.05% glutaraldehyde in 0.1 mol/L PO_4 buffer, treated with polyclonal antibody to GPIIIa (1:500 dilution), exposed to protein-A gold (5 nm), and then processed for electron microscopy as previously described.[16]

Since the reason for some of the technical modifications may not be immediately apparent, the rationale in brief is presented here. The NH_4Cl in the buffer wash quenches remaining aldehydes in the tissues[27] and thus prevents further fixation; the effect on total antigen survival is modest. Acetone dehydration allows survival of all of the antigens, whereas ethanol dehydration markedly decreases the survival of some antigens, particularly GPIIb and the GPIIb-IIIa complex. Coverslips should dry overnight or longer at room temperature to ensure adhesion through the entire immunohistochemical procedure. We found that trypsin digestion for longer than 15 minutes degraded the morphology and that only a brief trypsin exposure was necessary. Some antigens could be detected without trypsin digestion. We found overnight (4 °C) incubation with primary antibody to be the most convenient and reliable procedure; good results were also obtained with a four- to six-hour incubation at room temperature or a two-hour incubation at 37 °C.

The large amount of endogenous peroxidase poses a particular problem for bone marrow studies. We approached this problem by combining several techniques. We inhibited with methanol-H_2O_2 after the biotinylated secondary antibody to prevent interference with antigen-antibody reactions. We selected a lower concentration of these reagents than is generally used because higher concentrations had a tendency to loosen the sections. The addition of sodium azide, which inhibits animal but not plant peroxidases,[25] further improves the inhibition of endogenous peroxidase. The use of this combination usually results in complete inhibition of peroxidase in eosinophils and red cells. Weak persistence of reactivity in these cells is occasionally noted. We have also had some success using phenylhydrazine[28] in a preincubation prior to the primary antibody step. A consistent, apparently nonspecific, reaction was seen in mast cells using all of the primary antibodies. These cells have been shown to bind avidin complexes.[29] There was no other binding suggesting the presence of endogenous biotin[30] or avidin.[31]

RESULTS

The main structural components of human platelets and megakaryocytes have been well defined by ultrastructural studies in recent years.[32] Antibodies to a variety of platelet proteins have been produced in our laboratory and by others. Most of these proteins can be classified by their fine structural localization in platelets into three main groups: (1)

plasma membrane proteins, (2) α-granule matrix proteins, or (3) α-granule membrane proteins. Using antibodies against these antigens and the technique described previously, we were able to precisely localize the proteins within the megakaryocytes of well-preserved intact biopsy specimens of human bone marrow (Figs 1A through 1F). Control preparations showed no evidence of nonspecific staining with the exception of the mast cell labeling previously noted. The results are summarized in Table 1.

Staining With Antibodies and Platelet Plasma Membrane Proteins

GPIIIa. Polyclonal antisera against GPIIIa labeled an average of 99% (range, 97% to 100%) of morphologically identifiable bone marrow megakaryocytes (Figs 1A and 1B). Rare mononuclear cells, presumably megakaryocytic precursors, were also labeled. Staining in megakaryocytes was distributed throughout the cytoplasm but was heaviest on the plasma membrane. This pattern was most readily observed in thin sections (1 μm) at high antibody dilutions and is consistent with localization of the antigen in both the plasma membrane and the demarcation membrane system and/or rough endoplasmic reticulum. In some megakaryocytes, distinct staining around the nucleus suggestive of localization in the perinuclear cisterna could be seen (Fig 1B). The monoclonal antibody (AP-3) showed the same distribution.

The plasma membrane localization of GPIIIa on nonpermeabilized megakaryocytes has been confirmed by electron microscopical examination (Fig 2). Studies on permeabilized cells that will allow the labeling of intracellular structures are in progress.

There was no staining of other hematopoietic cells. The endothelial cells within the marrow showed moderate staining, and osteoclasts showed a striking plasma membrane reaction (Fig 3) with both monoclonal and polyclonal antibodies.

GPIIb. The monoclonal antibody (Tab) that recognizes GPIIb produced strong staining in an average of 97% (range, 89% to 100%) of morphologically recognizable bone marrow megakaryocytes (Fig 1C). Rare mononuclear cells, presumably megakaryocytic precursors, were also stained (inset, Fig 1C). The staining pattern was very similar to that for GPIIIa, ie, present throughout the cytoplasm, prominent on the plasma membrane, and occasionally perinuclear. There was no staining of other hematopoietic or marrow cells, including osteoclasts. Staining with polyclonal antisera against GPIIb showed the same distribution.

GPIIb-IIIa complex. The monoclonal antibody (T-10) that recognizes the GPIIb-IIIa complex produced strong staining in an average of 96% (range, 91% to 100%) of morphologically recognizable bone marrow megakaryocytes (Fig 1D). The localization of staining was similar to that observed with antibodies to GPIIIa and GPIIb. In unstained sections peroxidase was clearly visible along the nuclear membrane in a few megakaryocytes (Fig 1D), indicating that the antigen may be present in the perinuclear cisterna, a compartment of the rough endoplasmic reticulum. As with GPIIIa and GPIIb, diffuse cytoplasmic staining was also present. Rare cells consistent with megakaryocytic precursors were stained, but other hematopoietic cells were negative. There was no staining of other marrow elements. Osteoclasts and endothelial cells did not stain.

Staining With Antibodies to Platelet α-Granule Matrix Proteins

Thrombospondin. Monoclonal antibodies against thrombospondin produced a distinctly granular reaction throughout the cytoplasm of megakaryocytes, consistent with localization of the antigen in the α-granules (Fig 1E). On average, 98% (range, 91% to 100%) of morphologically recognizable megakaryocytes were reactive. Fewer presumed megakaryocyte precursors were labeled with the antibodies against thrombospondin and the other α-granule matrix proteins than with the antibodies against plasma membrane proteins. Rare cells showed perinuclear staining in uncounterstained preparations. No other marrow cells showed reaction product. The absence of staining in macrophages and endothelial cells is of interest since it has been shown that these cells can secrete thrombospondin in culture.[34] Jaffe and co-workers,[35] however, were unable to demonstrate thrombospondin in these cultured cells immunohistochemically.

Factor VIII–related antigen. Both monoclonal and polyclonal antibodies against VIIIR:Ag produced strong granular staining in an average of 95% (range, 84% to 100%) of morphologically recognizable megakaryocytes. The distribution was identical to that observed with thrombospondin. There was no labeling of other hematopoietic cells, but moderate endothelial cell staining was observed.

β-thromboglobulin. Polyclonal antisera against β-thromboglobulin produced a strong granular reaction similar to that observed with VIIIR:Ag and thrombospondin in 94% (range, 84% to 100%) of the megakaryocytes. There was some nonspecific background labeling, but other hematopoietic and marrow cells appeared to be negative.

Platelet factor 4. Polyclonal antisera against PF4 produced a granular reaction similar to that observed with the other antibodies to α-granule proteins. An average of 94% (range, 85% to 100%) of the megakaryocytes were positive. There was slight background staining with the antibody and weak staining of endothelial cells and fat cell membranes.

Fibrinogen. Polyclonal antisera against fibrinogen produced a strong granular reaction consistent with α-granule localization. There were somewhat fewer positive cells, 84%, and a wider range (60% to 99%) than with antibodies against the other α-granule proteins. There was strong staining of fibrin strands within tissue and vascular spaces. In addition, many fat cell membranes and endothelial cells appeared to be positive.

Staining With Antibodies to Platelet α-Granule Membrane Protein 140

Both polyclonal and monoclonal (S-12) antibodies developed against the α-granule membrane protein with a molecular weight of 140 kilodaltons produced strong staining of 95% (range, 82% to 99%) of morphologically recognizable marrow megakaryocytes (Fig 1F). The pattern of staining was very similar to that observed with the antibodies against platelet α-granule matrix proteins. Although the granules were generally distributed throughout the cytoplasm, the

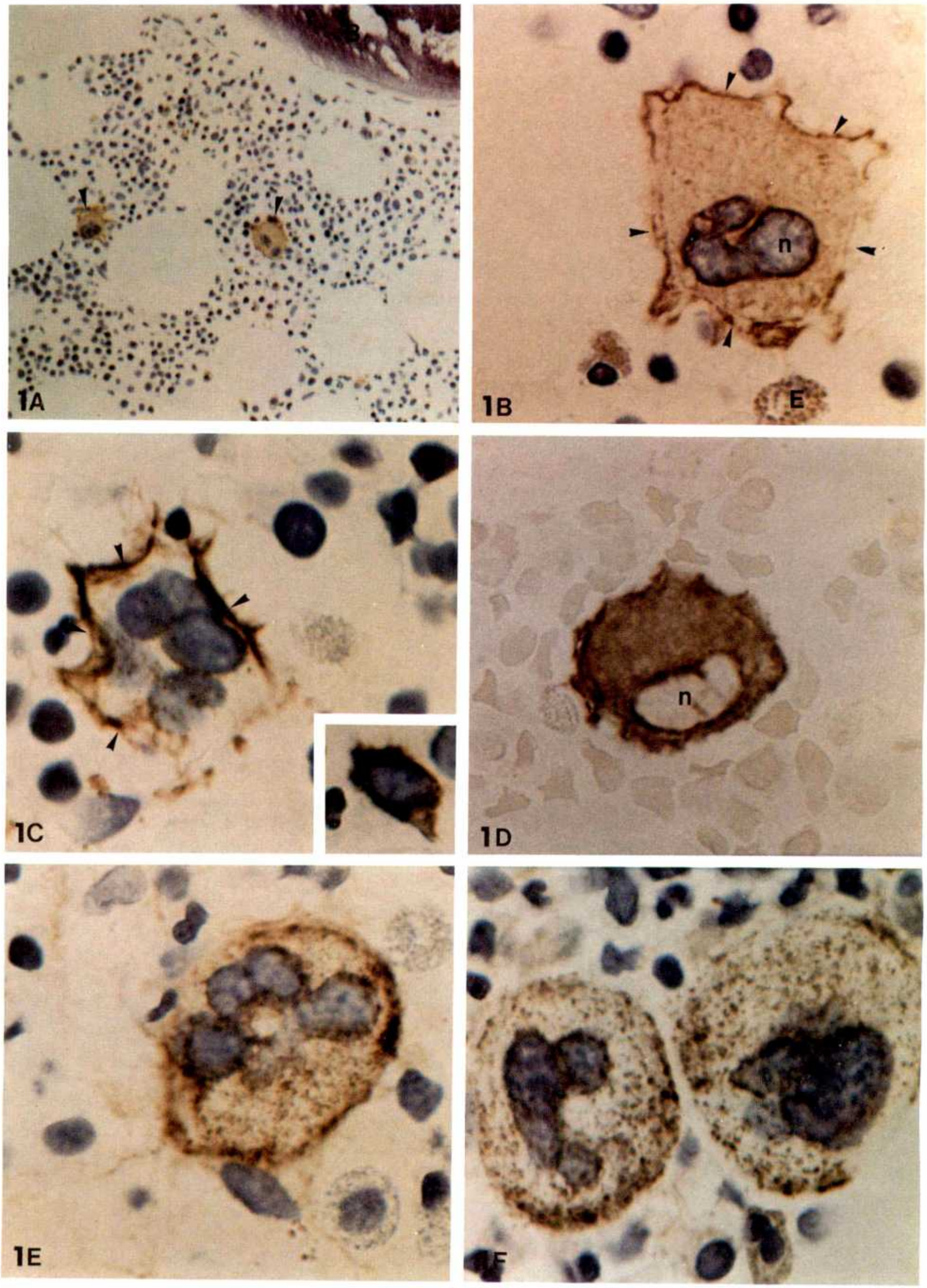
1A
1B
n
E
1C
1D
n
1E
1F

Table 1. Immunohistochemical Reactions in Megakaryocytes

Specimen No.	GPIIIa	GPIIb	GPIIb-IIIa	TSP	FVIIIR:Ag	βTG	PF4	Fib	GMP140	UEA
1	97/100	155/159	130/134	96/100	78/93	67/71	53/55	48/62	95/100	47/52
2	84/84	32/36	31/37	53/55	49/53	53/55	43/50	20/32	47/49	67/68
3	100/100	100/100	93/100	97/100	91/100	69/82	90/100	74/100	96/100	55/56
4	100/100	100/100	99/100	98/100	96/100	96/100	97/100	86/100	100/100	N/D
5	79/81	47/49	73/79	68/72	37/41	45/48	49/56	28/43	49/51	52/59
6	174/174	142/154	134/135	136/139	130/131	109/111	64/64	96/103	153/156	44/48
7	99/100	95/100	99/100	100/100	98/100	97/100	97/100	74/93	89/92	86/90
8	100/100	89/100	91/100	91/100	92/100	87/100	94/100	80/100	97/100	80/100
9	74/74	59/60	58/58	66/67	64/64	54/57	51/51	65/68	68/69	58/61
10	80/80	55/56	42/43	80/81	52/53	34/38	62/64	32/36	39/41	34/38
11	100/100	99/100	98/100	100/100	94/100	93/100	93/100	66/77	76/80	81/90
12	97/100	57/61	50/52	97/100	75/76	58/62	58/61	42/70	27/33	62/69
13	100/100	97/100	66/69	92/98	95/100	38/41	46/52	48/68	33/35	61/70
14	97/100	100/100	99/100	99/100	100/100	94/100	97/100	87/100	96/100	87/89
15	100/100	97/100	98/100	99/100	97/100	96/100	97/100	96/100	84/91	97/100
16	99/100	99/100	98/100	100/100	81/82	77/80	80/85	55/59	57/60	64/72
17	100/100	99/100	100/100	99/100	100/100	96/100	97/100	91/100	98/100	100/100
18	97/100	100/100	78/80	97/98	97/100	95/100	92/99	57/62	98/100	ND
19	99/100	100/100	98/100	98/100	97/100	92/96	72/73	83/84	98/100	56/56
20	100/100	99/100	95/100	100/100	92/100	96/100	85/100	78/100	99/100	ND
21	100/100	100/100	100/100	96/100	86/92	93/100	93/100	N/D	96/100	ND
22	98/100	99/100	93/100	99/100	53/62	88/99	89/100	55/67	71/81	ND
23	92/94	98/100	89/93	80/83	40/45	65/70	53/59	45/55	69/75	ND
24	100/100	91/95	95/100	88/90	74/77	96/100	62/64	96/100	60/68	ND
25	65/65	94/96	100/100	88/89	73/73	100/100	55/56	43/46	99/100	64/68
Average	99%	97%	96%	98%	95%	94%	94%	84%	95%	93%

Abbreviations: TSP, thrombospondin; Fib, fibrinogen; UEA, Ulex europaens; ND, not determined.

Results expressed as positive megakaryocytes over total megakaryocytes.

region beneath the plasma membrane was usually devoid of granules. This region was particularly striking when the sections were compared with serial sections labeled for the plasma membrane proteins in which the irregular interdigitations of the plasma membrane with the surrounding cells were well demonstrated (Figs 1B through 1D). Electron microscopy (Fig 2) also demonstrates the irregularities of the plasma membrane and the relatively α-granule–free zone beneath the membrane. This pattern is consistent with localization of GMP140 in α-granule membranes, which has been confirmed ultrastructurally in our laboratory using these same antibodies.[36] Occasional cells showed perinuclear staining similar to that observed with the antibodies to the membrane glycoproteins and α-granule matrix proteins. All other hematopoietic cells were negative. Some marrow endothelial cells were reactive.

Other Markers

The enzyme histochemical and lectin reactions were consistent, but not specific, markers in human megakaryocytes.

Fig 1. Biopsy specimens of normal human bone marrow embedded in plastic and tested immunohistochemically for a variety of platelet antigens. The brown peroxidase reaction is present at sites of antigen localization. Scattered eosinophils show partial persistence of endogenous peroxidase. The cells were counterstained with hematoxylin except in Fig 1D. Sections A through D show the distribution seen with the platelet plasma membrane glycoproteins used in this study. The staining was heaviest on the plasma membrane with lesser amounts distributed through the cytoplasm. Figures 1A and 1B show the typical appearance, whereas Figs 1C and 1D show minimal and maximal reactions respectively. The percentage of cells displaying these variations can be manipulated by thickness of section and antibody titer as noted in the text. Figures 1E and 1F show the typical localization patterns with antibodies to α-granule matrix and membrane proteins. Less variability was noted with these antibodies. (A) Megakaryocytes (arrows) labeled with the polyclonal antibody to IIIa show brown staining that is easily identified even at this low magnification (abbreviation: B, bone). Original magnification ×200. (B) At higher magnification it is apparent that the megakaryocyte plasma membrane (arrows) is strongly reactive, whereas the cytoplasm shows a lighter diffuse reaction. A suggestion of perinuclear staining is also present, but difficult to see with the nucleus (n) counterstained. The endogenous peroxidase is only partially inhibited in the eosinophil (E). Original magnification ×1,260. (C) In this thin section labeled with monoclonal antibody (Tab) to GPIIb, almost all of the reaction product is deposited on the plasma membrane (arrows). The typical scalloped borders of the cell are well visualized. The inset shows an immature mononuclear cell, presumably a megakaryocyte precursor, with strong cytoplasmic and membrane staining. Original magnification ×1,260. (D) Monoclonal antibody (T-10) against the GPIIb-IIIa complex strongly stains a megakaryocyte; the brown reaction product is present on the irregular plasma membrane as well as in the cytoplasm. In this section without counterstain the nuclear membrane staining is clearly visible. Original magnification ×1,260. (E) A distinctly granular reaction is present throughout the cytoplasm with the monoclonal antibody to thrombospondin. In this particular cell, there is an increased concentration of granules near the periphery of the cell. The plasma membrane is not stained. Original magnification ×1,260. (F) Monoclonal antibody (S-12) to GMP140 produces a brown granular reaction product distributed in a pattern very similar to that seen with the α-granule matrix proteins. The plasma membrane is not stained. Original magnification ×1,260.

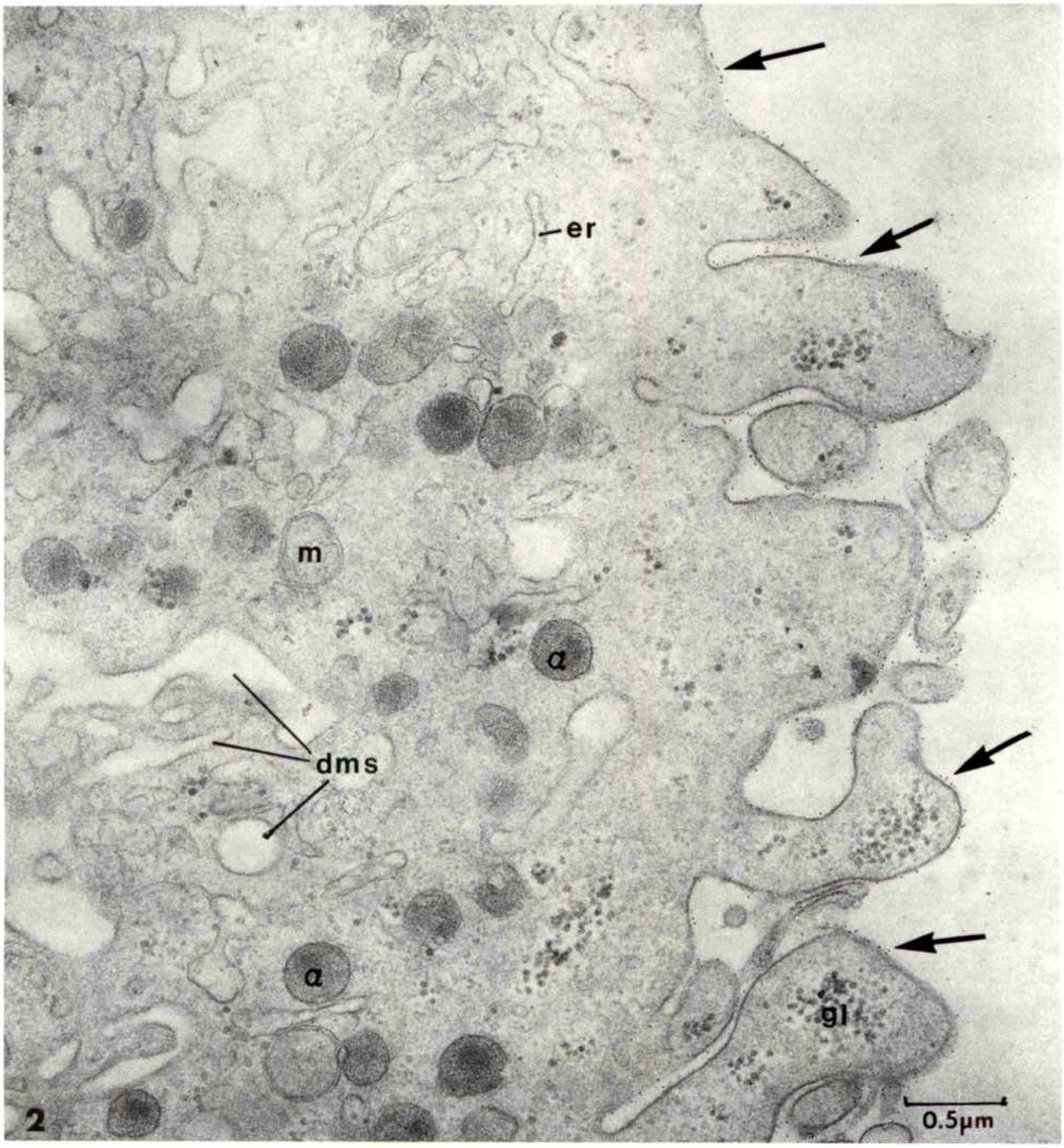

Fig 2. Transmission electron micrograph of part of the cytoplasm of a human megakaryocyte illustrating the presence of GPIIIa (arrows) on the plasma membrane as revealed by an immunogold probe. The plasma membrane is quite irregular. Note that the immunogold label does not penetrate into the demarcation membrane system despite the fact that this membrane system has been shown to be open to the extracellular space when using small tracers such as tannic acid.[33] This may be due to the larger size of the primary antibody or the secondary immunogold probe. Abbreviations: α, α-granules; m, mitochondrion; er, endoplasmic reticulum; dms, demarcation membrane system; gl, glycogen. Original magnification ×31,000.

The lectin Ulex europaeus produced strong, diffuse cytoplasmic labeling in most of the megakaryocytes, but also stained the membranes of early myeloid cells and later erythroid precursors and red cells. All endothelial cells showed strong staining. Most megakaryocytes were positive for PAS, α-naphthyl acetate esterase, and acid phosphatase. The PAS reaction was abolished by digestase; the acid phosphatase reaction was completely inhibited by tartaric acid; and the α-naphthyl acetate esterase reaction was partially inhibited by sodium fluoride. The enzyme α-naphthyl butyrate esterase was present but weak. (This observation differs from our previous observation[12] and is a result of a change in the fixation procedure.) It was also partially inhibited by sodium fluoride. No megakaryocytes were reactive for alkaline phos-

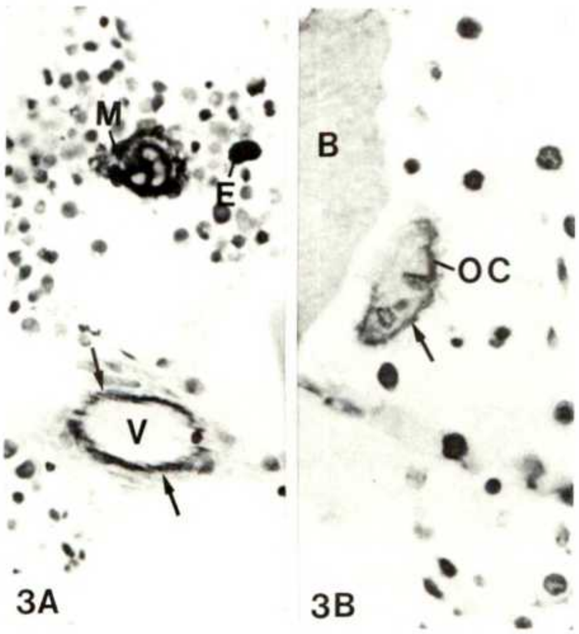

Fig 3. Biopsy specimens of human bone marrow embedded in plastic and reacted immunohistochemically for the platelet membrane GPIIIa. (A) The endothelial cells of a small vein (V) stain strongly (arrows) as does an adjacent megakaryocyte (M) and an incompletely inhibited eosinophil (E). (B) A multinucleate osteoclast (OC) located characteristically adjacent to bone (B) shows a moderately strong (arrows) reaction on the plasma membrane. Original magnification of A ×360, of B ×575.

phatase, 5-nucleotidase, or ATPase. Antibody against the human Ia antigen (HLA-DR) showed no reactivity with morphologically identifiable megakaryocytes.

DISCUSSION

Although normal mature megakaryocytes are among the most readily recognizable of bone marrow cells, abnormal and immature cells of megakaryocytic origin have been much more difficult to recognize. This has limited our understanding of their role in certain pathologic states. In murine bone marrows, the enzyme acetylcholinesterase is specific for megakaryocytes and has been very useful in identifying them.[37] Unfortunately, an enzyme specific for human megakaryocytes has not been identified. Recent efforts to develop antibody probes for proteins unique to platelets and megakaryocytes have raised hopes for a reliable tool for identifying human megakaryocytes. Use of these probes, however, has been limited to identifying megakaryocytes in smears,[1] in culture,[38] in concentrated bone marrow aspirates,[2,3] and in flow cytometry systems.[39] The intact biopsy specimen has presented greater difficulties. Many of the pathologic states in which it would be desirable to identify megakaryocytes are complicated by fibrosis.[40] The technique described in this paper is applicable to any bone marrow disorder including those with fibrosis. This has been confirmed in preliminary studies in our laboratory that have demonstrated the ability to identify megakaryocytes in a variety of myeloproliferative syndromes.

In the present study we have shown that a variety of antibody probes can be used to identify normal megakaryocytes in intact biopsy specimens. With the exception of fibrinogen, more than 90% of mature megakaryocytes were labeled by antibodies directed against platelet α-granule constituents or platelet membrane glycoproteins. Similar results have been obtained with antibodies against fibrinogen, VIIIR:Ag, myosin, and membrane GPIb and GPIIIa using immunofluorescence techniques.[38] One should note that some morphologically normal megakaryocytes in our specimens failed to label. Rabellino et al[2,3] observed a similar lack of labeling in some megakaryocytes. This finding suggests several possibilities, including variability in the production of platelet proteins by normal megakaryocytes, loss of these substances by degranulation, and technical variability. These possibilities should be considered when one is examining megakaryocytes in pathologic states.

Rare small mononuclear cells, probably megakaryocyte precursors, were also labeled with many of the antibody probes. Using immunofluorescence techniques and antibodies against VIIIR:Ag, PF4, and membrane GPIb, GPIIb, and GPIIIa, Rabellino et al[3] also detected label in a small number of mononuclear cells, which they regarded as early megakaryocytes. Using immunofluorescence and immunogold procedures, Breton-Gorius and associates have detected certain glycoproteins on the plasma membranes of immature megakaryocytes in both normal and abnormal human bone marrow specimens[41,42] and tissue culture cells.[38] Detection of such early marrow megakaryocytes in intact biopsy specimens should prove extremely useful in the diagnosis of pathologic conditions involving the megakaryocyte cell lineage.

An additional aspect of our technique that should be emphasized is the resolution allowed by the thinness of the plastic sections. For example, the granular appearance of antigen for GMP140 and the α-granule matrix proteins is consistent with their fine structural localization in α-granules.[16,36] In addition, the perinuclear localization of certain antigens is consistent with the synthesis of these proteins in the rough endoplasmic reticulum (RER). These results suggest that the technique may be valuable as a preliminary step for organelle localization of antigens in studies where sampling by electron microscopy is difficult.

We did not find GPIIb-IIIa, GPIIb, or GPIIIa in monocytes or macrophages in bone marrow. This is in agreement with the findings of Clemetson et al.[43] The identification of GPIIIa in human osteoclasts was an unexpected finding, as was the identification of GMP140 in some endothelial cells. The finding of GPIIIa in endothelial cells confirms the observation of Thiagarajan et al.[44] Neither osteoclasts nor endothelial cells showed evidence of staining for the GPIIb-IIIa complex. The presence of these proteins in endothelial cells suggests that they play a more complex role than had been previously suspected. The finding of the GPIIIa in osteoclasts is a particularly interesting observation that needs further study.

The data presented here demonstrate that mature and immature megakaryocytes can be reliably characterized in intact bone marrow biopsy specimens by use of markers for a wide spectrum of platelet proteins. Although the nonimmunologic markers that we examined may have clinical uses in suggesting megakaryocytic differentiation, their utility is considerably hampered by their lack of specificity. However, the ability to combine enzyme histochemical and immunohistochemical techniques in sections from a single specimen may be clinically useful. The technique described in this paper should improve quantitative and qualitative evaluation of human megakaryocytes in a variety of clinical settings. The ability to demonstrate several antigens may prove to be particularly useful in pathologic conditions in which the cells' ability to produce a normal complement of proteins may have been altered, eg, in the myeloproliferative[45,46] and myelodysplastic syndromes (unpublished observations: Beckstead, Doyle [University of Wisconsin], and Bainton). Furthermore, the ability to apply these markers to pathologic states with marrow fibrosis should increase our understanding of the role of megakaryocytes in a variety of diseases. This technique has also been helpful in examining a variety of other tissues, including lymph nodes,[47] kidneys,[48] and skin (Kaposi's sarcoma).[49] It may allow us to broaden our understanding of the function of some proteins through easy application to a broad range of tissues. For example, we have recently noted that the antigen PF4 appears to be present in the bile ducts of human liver and that GPIIIa and GMP140 are present in endothelial cells in tissues besides bone marrow.

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Immunoregulation of B Lymphocyte Colony Formation by T Cell Subsets in Patients With Chronic Lymphocytic Leukemia

By Louis A. Fernandez, J. Michael MacSween, and G. Ross Langley

Normal B lymphocytes are activated, proliferate, and then differentiate into plasma cells and secrete immunoglobulin (Ig). We have reported that chronic lymphocytic leukemia (CLL) T4 cells help and CLL T8 cells lack suppressor effects on Ig synthesis by normal B cells (*Blood* 62:767, 1983). We have now explored the earlier phase, proliferation, using B cell colony formation in semisolid media. B lymphocyte colonies from normal individuals and from patients with CLL were grown in 0.3% agarose overlayed with T cells or T cell subsets and the B cell mitogen staphylococcal protein A. Enriched T cells, OKT4 or OKT8, were obtained either by sheep erythrocyte rosettes or depletion of OKT8 or OKT4 cells by monoclonal antibody or complement, respectively. Twenty thousand B cells from normal subjects yielded 65 ± 9, 64 ± 7, and 19 ± 6 colonies with autologous unfractionated T-, OKT4-, or OKT8-positive cells, respectively. This compared to 29 ± 11, 81 ± 11, and 15 ± 4 colonies from patients with CLL with added autologous unfractionated T-, OKT4-, or OKT8-positive cells. To determine whether the fewer number of colonies in both normal subjects and patients with CLL with OKT8-positive cells was due to suppression or lack of help, the number of OKT4-positive cells was held constant, and OKT8-positive cells were added in increasing numbers. No suppression of colony formation could be demonstrated. Furthermore, the addition of increasing numbers of concanavalin A (Con A)-activated OKT8-positive cells did not suppress colony formation. These results suggest that the CLL T cell subsets behave in a functionally similar manner to normal T cell subsets, namely, (1) that normal and CLL B cell colony growth is helped by OKT4 cells; and (2) in contrast to immunoglobulin secretion by B cells, neither normal nor CLL OKT8 cells, unstimulated or activated by Con A, suppress B cell colony growth.

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NORMAL B LYMPHOCYTES are activated, proliferate, and then differentiate into plasma cells and secrete immunoglobulin.[1] We have reported that chronic lymphocytic leukemia (CLL) T4 cells help and that CLL T8 cells lack suppressor effects on normal B cell immunoglobulin secretion.[2] We have now explored the earlier phase, proliferation, using B cell colony formation in semisolid media. In this system small lymphocytes from normal individuals transform into blast cells and form colonies of 50 to 500 cells.[3,4] Although some of the cells in normal colonies are mature plasma cells, many remain blast-like, whereas B cell colonies from patients with CLL do not contain mature plasma cells.[5] The requirement for T cells and their subsets for colony formation from a single clonogenic precursor can be determined in this system.[6]

Normal B cells can be stimulated in liquid culture with a variety of B cell mitogens (pokeweek mitogen [PWM] and lipopolysaccharide [LPS]), and this can be quantitated by incorporation of tritiated thymidine and/or secretion of immunoglobulin.[7–9] However, we[2] and others[10–12] have observed that, in response to these B cell mitogens, CLL B cells either do not proliferate or else the fraction of cells that do proliferate is so small that incorporation of tritiated thymidine is not sensitive enough to pick up proliferative activity. In contrast, the B cell colony assay circumvents this problem since both normal and CLL B cell colonies can be grown using similar conditions.[3,5,13–15] T cells and staphylococcal protein A (SPA) are required for colony formation, or alternatively, mitomycin-treated T cells and supernatants obtained from normal T cells stimulated with phytohemagglutinin (PHA) (PHA-TCM). The B cell colonies grown from patients with CLL have the same phenotype as the peripheral blood leukemic cell, and "residual normal B cells" within the leukemic clone do not contribute appreciably to the colony growth.[16] Thus, B cell colony growth in CLL reflects the proliferative potential of the malignant cells. We have therefore used this assay to study some of the characteristics of leukemic B cell proliferation.

MATERIALS AND METHODS

Patients The studies were performed on 17 patients with untreated CLL, ten males and seven females whose ages ranged from 49 to 83 years. The diagnosis of B cell CLL was based on an absolute lymphocytosis of greater than 10,000 cells/μL on two successive occasions without any known cause, exhibiting infiltration of the bone marrow by morphologically mature lymphocytes. Surface markers showed that the majority of lymphocytes were of the B cell type, as demonstrated by the presence of small amounts of membrane immunoglobulin[17] and receptors for the C3 component of complement.[18] According to the classification of Rai et al,[19] nine were stage 0 (bone marrow and blood lymphocytosis), three were stage I (lymphocytosis with enlarged nodes), four were stage II (lymphocytosis with enlarged spleen), and one was stage III (lymphocytosis and thrombocytopenia) Thus the majority were in early stages of the disease

Lymphocyte colonies Mononuclear cells were obtained by centrifuging heparinized venous blood on Ficoll-Hypaque. The cells were then rosetted with sheep erythrocytes and centrifuged over Ficoll-Hypaque, and T cells were recovered by lysing the sheep erythrocytes with 0.87% ammonium chloride.[2,20] These T cells, either untreated or treated with mitomycin C, 50 μg/mL, for 30 minutes at 37 °C, were washed three times and used as feeder cells. The mononuclear cells depleted of T cells were allowed to adhere to Petri dishes containing RPMI 1640 and 20% autologous plasma for

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one hour at 37 °C. The nonadherent cells, enriched mainly for B cells, were recovered, washed, and resuspended in RPMI 1640 with 10% fetal calf serum.[3,13,15] The enriched B cells from normal individuals and patients with CLL were contaminated with 5% ± 3% and 2% ± 1% T cells, respectively.

B cells were cultured with SPA[6] or PHA-TCM as a mitogenic stimulus. For SPA-induced colonies, B cells were suspended in 0.1 mL of 0.3% agarose containing RPMI 1640, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 100 µg/mL kanamycin, and 2.5 µg/mL fungazone. The layer was allowed to harden at room temperature for 30 minutes. It was then overlayed with RPMI containing the aforementioned antibiotics, 24% fetal calf serum, various concentrations of T cells, and SPA. The microtiter plates were covered and placed in a plastic bag. These were then placed in a 5% incubator with a moist atmosphere for five to nine days.

For colonies generated by PHA-TCM, enriched B cells were mixed with mitomycin-treated T cells in various ratios, suspended in 20% PHA-TCM, 8% methyl cellulose, and growth medium and gently mixed on a vortex mixer.[3,13,15] Triplicate samples of 0.1-mL aliquots were placed in flat-bottom microtiter plates. The plates were covered and placed in plastic bags and then placed in a 5% incubator with a moist atmosphere for five to nine days. The plates were then examined for B cell colonies, defined as aggregates of greater than 50 cells. These cells were stained for surface immunoglobulins and did not rosette with sheep erythrocytes. Appropriate controls were run for each experiment, namely, T and B cells alone and combinations of T and B cells without mitogens.

PHA-TCM was prepared by stimulating T cells (as obtained in the preceding procedure) with 1% PHA in RPMI 1640 for three days in a 5% CO atmosphere.[13] Supernatants of such cultures from three individuals were pooled and sterilized by passing them through a millipore filter and storing them at −4 °C.

Enrichment of T helper and suppressor cells using monoclonal antibodies Enriched OKT4 and OKT8 subsets of T cells were obtained as previously described[2] Briefly, 10 to 20 million cells suspended in 1 mL of RPMI and 5% fetal calf serum (GIBCO, Burlington, Ontario, Canada) were mixed with 10 µL of OKT4 or OKT8 monoclonal antibodies (Ortho Diagnostics, Don Mills, Ontario, Canada) and held for 0.5 hours at 0 °C. Then, 300 µL of rabbit complement (Cedarlane, Hornby, Ontario, Canada, batch 6045) were added, and the tubes were placed on a rotator for one hour at 37 °C. These cells were washed three times with RPMI 1640 containing 5% fetal calf serum. Aliquots of cells were stained with fluorescein-conjugated antibodies to OKT4 or OKT8. Less than 1% of the T cells depleted of T4 or T8 subsets displayed a staining of their respective fluoresceinated antibodies although the OKT4 cells and OKT8 cells were enriched to a mean of 76% and 79%, respectively.

Concanavalin A–activated T8 cells Five million OKT8 cells were suspended for two hours at 37 °C in 1 mL of RPMI 1640 containing L-glutamine, 2 mmol/L, and concanavalin A (Con A), 10 µg/mL. This concentration was found in our laboratory to be optimal for generating suppressor cells.[21] The cells were then washed three times with medium before use.

Mixed lymphocyte reaction. The mixed lymphocyte reaction (MLR) was performed as described before.[22] Responder cells consisted of T cells, T cell subsets, and Con A–activated T8 cells in different ratios. Fifty thousand responder cells were mixed with an equal number of mitomycin C–treated (50 µg/mL) mononuclear cells from a panel of four donors (stimulator cells). These cells were suspended in RPMI 1640 containing 10% fetal calf serum and placed in microtiter plates for seven days at 37 °C in a 5% CO_2 atmosphere. The evening before harvesting the cells, 0.5 µCi of tritiated thymidine (specific activity, 15.1 mmol/L) was added. The next day the cells were harvested onto glass filters using a Titertek harvester (Flow Laboratories, Mississauga, Ontario, Canada). The filters were then placed in vials containing scintillating fluid and the radioactivity determined in a beta scintillation spectrometer. Statistical analysis was performed using Student's *t* test for paired samples and group means.

RESULTS

Appropriate controls showed that B cell colonies were not formed in the absence of mitogens, B cells, or T cells. No colony growth was observed when B cells were cultured either alone (n = 6 normals and 11 patients) or with mitogens without T cells (n = 4 normals and 7 patients). Table 1 shows the number of colonies generated simultaneously from the peripheral blood B cells of ten patients with CLL and normal individuals. These colonies were grown in semisolid media using mitomycin-treated T cells and normal T cell supernatants. There were significantly fewer colonies from B cells of patients with CLL than normal individuals ($P < .025$). Enrichment of T cells with OKT4-positive cells resulted in more colonies than enrichment with OKT8 cells (normal, $P < .05$; CLL, $P < .025$). B cell colony growth was decreased when CLL OKT4 cells were used compared to unfractionated CLL T cells; however, this difference was not significant. When these CLL OKT4 cells were added to normal B cells, 102 ± 22 B cell colonies were obtained, compared to 90 ± 15 with unfractionated CLL T cells, suggesting that CLL OKT4 cells provide normal helper activity for the growth of normal B cell colonies.

Since B cell colonies can be generated using different mitogens, we also grew colonies using SPA as the mitogen (Table 2). The results were similar to those obtained using PHA-TCM, showing fewer colonies from cells of patients with CLL than normal individuals ($P < .05$) and more colonies with enrichment of T cells by OKT4-positive cells than with enrichment by OKT8 cells (normal, $P < .001$; CLL, $P < .05$). The help provided by the CLL OKT4 cells for the leukemic B cell colony growth was increased in comparison with the whole T cell population although the increase was not significant, suggesting that the T4 cells did not have an intrinsic defect in helper activity.

To determine whether the OKT8 cells suppressed colony formation or merely failed to provide adequate help, several experiments were carried out (Tables 3 to 6). When the total number of T cells was kept constant but the numbers of OKT4 and OKT8 varied so that the ratios of T4 to T8 were 1:0, 1:1, 1:2, and 0:1, the number of B cell colonies was decreased both in normal individuals and patients with CLL as the number of T4 cells was decreased and T8 cells

Table 1. Numbers of B Cell Colonies Generated in Semisolid Media Using PHA-TCM as a Mitogen

Source of B Lymphocytes	No. B Cell Colonies Generated Using Autologous		
	T Cells	T4 Cells	T8 Cells
Normal individuals (n = 10)	149 ± 33*	120 ± 28	90 ± 28
Patients with CLL (n = 10)	74 ± 18	51 ± 11	20 ± 5

*Mean ± SEM.

Table 2. Numbers of B Cell Colonies Generated in Semisolid Media Using SPA as a Mitogenic Stimulus

Source of B Lymphocytes	No. B Cell Colonies Generated Using Autologous		
	T Cells	T4 Cells	T8 Cells
Normal individuals			
(n = 9)	65 ± 9	64 ± 7	19 ± 6
Patients with CLL			
(n = 5)	29 ± 11	81 ± 11	15 ± 4

Table 3. Effect of Different Proportions of Autologous T Cell Subpopulations on the Number of Colonies Generated in Semisolid Media Using SPA as a B Cell Mitogen

Source of B Lymphocytes	No. Colonies Generated Using			
	T4 140,000 T8 0	T4 70,000 T8 70,000	T4 47,000 T8 93,000	T4 0 T8 140,000
Normal individuals				
(n = 7)	76 ± 26*	40 ± 7	18 ± 6*	28 ± 5
Patients with CLL				
(n = 7)	94 ± 23†	54 ± 11†	65 ± 14	43 ± 8

*Significantly different from each other, $P <$ 05.

†Significantly different from each other, $P <$ 05.

increased (Table 3). A significant decrease in B cell colonies was seen in normal individuals when the T4:T8 ratio was 1:2 ($P < .05$) and in patients with CLL when the T4:T8 ratio was 1:1 ($P < .05$). Similar results were obtained when allogeneic T cells replaced autologous T cells using normal T cells mixed with CLL B cells and vice versa (Table 4).

Since the decrease in number of B cell colonies could be due to suppression by OKT8 cells or to decreased help from fewer numbers of OKT4 cells, we added increasing numbers of OKT8 cells to a constant number of OKT4 cells. As a control, increasing numbers of unfractionated T cells were added to a constant number of OKT4 cells. As seen in Table 5, there was no significant suppression of B cell colony numbers either in normal individuals or patients with CLL.

To evaluate the possibility that OKT8 cells did not suppress colony formation by virtue of being in an inactive state, they were stimulated with Con A, and the various numbers were added to a constant number of OKT4 cells (Table 6). The Con A–stimulated OKT8 cells from both normal individuals and patients with CLL failed to suppress B cell colony formation significantly, although, as expected, they were quite capable of suppressing MLR (Table 7).

Table 4. Effect of Different Proportions of Allogeneic T Cell Subpopulations on the Number of Colonies Generated in Semisolid Media Using SPA as a B Cell Mitogen

Source of B Lymphocytes	No. Colonies Generated Using			
	T4 140,000 T8 0	T4 70,000 T8 70,000	T4 47,000 T8 93,000	T4 0 T8 140,000
Normal individuals				
(n = 7)	105 ± 20	65 ± 10*	73 ± 23*	63 ± 5
Patients with CLL				
(n = 7)	79 ± 22	58 ± 11	47 ± 15	29 ± 9*

*Significantly different from the corresponding first column, $P < .05$.

Table 5. Effect of Increasing Numbers of T8 Cells and a Constant Number of T4 Cells on the Number of B Cell Colonies Generated In Semisolid Media Using SPA as a Mitogen

Source of B Lymphocytes	No B Cell Colonies Generated Using Autologous			
	T4 140,000 T8 0	T4 140,000 T8 25,000	T4 140,000 T8 50,000	T4 140,000 T8 100,000
Normal individuals				
(n = 8)	62 ± 7	50 ± 10	58 ± 7	68 ± 7
Patients with CLL				
(n = 4)	85 ± 13	56*	49 ± 20	73 ± 16

*n = 1.

DISCUSSION

These studies show that the T cells required for colony formation of cultured B cells from normal individuals can be replaced by the T4 but not the T8 subset of cells. These results are similar to those reported by Whisler et al,[6] although they suggested that the decreasing number of B cell colonies resulting from decreasing ratios of T4:T8 cells was in keeping with suppression by the T8 cells. However, when we maintained the number of T4 cells constant and increased the T8 cells fourfold, there was no suppression of colony formation in either normal individuals or patients with CLL. Other investigators have reported similar data in normal individuals.[23] Any further increase in the numbers of T8 cells would take the ratio of T4:T8 out of the range observed in blood of normal individuals. Our interpretation therefore is that an absolute number of T4 cells and T4:B cell ratio is essential for colony formation, and the previous observations suggesting suppression are probably due to a decreased number of helper T cells. In this respect, the effect of normal T8 cells on colony formation differs from their effect on the latter phases of B cell maturation and immunoglobulin secretion, which they suppress.[2,24]

Con A activation of the OKT8 subset sufficient to cause a 50% suppression in the MLR did not suppress B cell colony growth either. Similar data were obtained when B cell colonies were grown in methylcellulose using mitomycin-treated T cells and PHA-TCM (data not shown). The normal T cell subsets behaved in a functionally similar manner when they were placed in culture with CLL B cells. When unfractionated, autologous T cells were used to provide help for B cell colonies, fewer colonies were obtained from patients with CLL than normal. A possible explanation for this observation is the known decreased proportion of the T4 subset in the unfractionated T cell population of CLL.[25,26]

Table 6. Effect of Con A–Activated OKT8 Cells on the Generation of B Cell Colonies

Source of Lymphocytes	No. B Cell Colonies Generated Using			
	T4 140,000 CT8 0	T4 140,000 CT8 25,000	T4 140,000 CT8 50,000	T4 140,000 CT8 100,000
Normal individuals				
(n = 4)	77 ± 8	67 ± 12	55 ± 4	63 ± 8
Patients with CLL				
(n = 4)	85 ± 13	74*	55 ± 2	64 ± 11

Abbreviation: CT8, Con A–activated OKT8 cells

*n = 1.

Table 7. Effect of Con A–Activated OKT8 Cells on Proliferative Responses in MLR Expressed as Counts per Minute

	Proliferative Responses in MLR (cpm)							
Source of Lymphocytes	MLR Control (T/T)*	MLR Test (T/Normal* Pooled Lymphocytes)	Number of OKT8 Cells Added			Number of Con A–Activated OKT8 Cells Added		
			10,000	25,000	50,000	10,000	25,000	50,000
Normal Individual	2,927	45,070	40,616	40,733	27,583	35,681	27,154	17,880
CLL 1	1,832	62,666	73,661	53,911	45,341	58,051	49,473	22,295
CLL 2	ND	4,864	ND	ND	ND	3,075	1,941	ND

Abbreviation: ND, not determined.

*Stimulators/responders.

In general, the functional activity of CLL T cell subsets for CLL or normal B cell colony formation was similar to those described for normal T cells. When methylcellulose and normal PHA-TCM were used, the CLL OKT4 subset was not as effective as unfractionated T cells in generating CLL B cell colonies, suggesting an OKT4 helper defect. However, this was not seen when CLL OKT4 cells were added to normal B cells stimulated with PHA-TCM or to CLL B cells stimulated with SPA. In fact, in the latter system the number of cell colonies generated by OKT4 cells was increased in comparison with unfractionated T cells. The reason for this is not clear; however, in general there was no lack of help in generating B cell colonies by CLL OKT4 cells.

We have shown previously that CLL OKT4 cells provided normal help to normal B cells in secreting immunoglobulin and that, unlike normal T8 cells, CLL OKT8 cells did not suppress this helper activity of the CLL OKT4 cells.[2] In the B cell colony assay, however, although both normal and CLL OKT4 cells provide help for colony formation, the OKT8 subset of cells in both cases was unable to suppress B cell colony numbers, and therefore we conclude that both OKT4 and OKT8 cells from patients with CLL function similarly to normal cells in this assay.

Our data suggest that proliferation of CLL B cells to form colonies is not impaired when adequate help is provided. We have also shown that both normal and CLL B cell colony formation is not suppressed by T cells. However, proliferation of B cells may be suppressed by other mechanisms such as immunoglobulin binding to Fc receptors.[27,28] Further experiments are being conducted to delineate the mechanism of suppression of normal B cells and then to apply these to abnormal states such as CLL.

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An Ultrastructural Morphometric Analysis of Platelet Giant and Fusion Granules

By Claire M. Payne and Lewis Glasser

Our purpose in this study was to establish ultrastructural morphometric criteria that can be used to define pathologic giant and fusion platelet granules and to determine whether patients with neoplastic myeloproliferative disorders (MPD) can be distinguished from other patients. We have morphometrically analyzed 2,391 giant and fusion granule profiles from 46 patients with neoplastic MPD, 127 other diseased control subjects, and 30 normal subjects using a computerized image analyzer. The largest granule profile observed in normal subjects had an area of 0.51 μ^2 and a perimeter of 3.21 μ. The most irregularly shaped of the large granule profiles photographed from normal subjects had a form factor (FF) value of 0.31. FF values indicate the degree of deviation of a given granule contour from a circle and is expressed by the formula $4\pi A/P^2$. A pathologic granule profile was then defined as a granule that exceeded any of these limits. Fifty-seven percent of the patients with neoplastic MPD were determined to have abnormal platelet granule profiles using the aforementioned morphometric criteria. It was determined, however, that morphometrically defined giant- and fusion-type granule profiles were nonspecific and were also found in 20% of the other diseased patients with no clinical evidence of an underlying neoplastic MPD. Morphometry has allowed us to define the upper limits of normal for the area and perimeter of individual platelet granules. Morphometrically defined giant fusion granules were determined to be more prevalent in the neoplastic MPD group, but because of their nonspecificity, may only have diagnostic significance for the individual patient in specific clinical settings. The pathogenesis of platelet fusion granules is discussed.

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ELECTRON MICROSCOPY has made a significant contribution to our understanding of the normal and defective human platelet.[1–5] Ultrastructural defects involving platelet granules have been well documented and include a deficiency or absence of dense bodies (Hermansky-Pudlak syndrome,[6] storage pool disease[7–9]) or α-granules (gray platelet syndrome[10,11]). An ultrastructural description of giant granules was first reported in 1960 in Willebrand-Jürgens disease.[12] Fusion granules (giant, irregularly shaped granules) resulting from an apparent fusion of normal-sized α-granules[12] have since been reported to be characteristic of platelets obtained from patients with preleukemia and myelomonocytic leukemia (MML).[13–17] If platelet fusion granules were specific, the diagnostic import would be similar to Auer rods, which are fusion granules found in neoplastic granulocytes. Platelet giant granules have, however, been reported, on a subjective basis, in normal human platelets,[1–3,14,18] platelets from patients with nonneoplastic hematologic diseases,[12,19–25] hairy cell leukemia,[26] and neoplastic myeloproliferative disorders (MPD) in addition to preleukemia and MML.[18,20,21,27–33]

Electron microscopic examination of normal platelets in our laboratory revealed occasional granule profiles that also appear fused, irregular in shape, and somewhat larger than the majority of other granule profiles. A qualitative assessment of what constitutes an abnormal granule has hitherto been subjective and does not adequately distinguish normal and pathologic granules. In this study, we have used a computerized image analyzer to morphometrically analyze 2,391 giant and fusion granule profiles from 46 patients with neoplastic MPD, 127 other diseased control subjects, and 30 normal subjects. Our purpose was to establish ultrastructural morphometric criteria that can be used to define the pathologic nature of platelet giant and fusion granule profiles and to determine whether patients with neoplastic MPD can be distinguished from other patients.

MATERIALS AND METHODS

Patient Selection

Normal subjects. Thirty adult volunteers from our faculty/staff who showed no evidence of an underlying hematologic or nonhematologic disorder were selected.

Steroid-treated normal subjects. This group was chosen because it represents a simple stress-related effect that could be used as a baseline for the stress related to diseased states in general. We have shown in a previous study that a single dose of dexamethasone had an effect on platelet ultrastructure.[34] We have examined this same material in the present study to determine whether steroids had any specific effect on platelet granules Briefly, 12 adult volunteers were given a single dose of oral dexamethasone (4 mg/m^2 of body surface area). Presteroid and poststeroid (nine to 13 hours after ingestion) blood samples were then analyzed.

Nonneoplastic, diseased control groups. This selection of patients consists of the following clinical groups.

1. Nonneoplastic platelet abnormalities: 40 patients with reactive thrombocytosis, eight patients with thrombocytopenia, four patients with congenital platelet disorders (two patients with the May-Hegglin anomaly, one patient with the Bernard-Soulier syndrome, and one patient with storage pool disease), and two patients with platelet satellitism
2. Nonneoplastic WBC abnormalities. reactive (five patients with eosinophilia, six patients with a granulocytic leukemoid reaction, two patients with monocytosis, and seven patients with lymphocytosis) and congenital (one patient with each of the following disorders: Chédiak-Higashi syndrome (CHS), Pelger-Huët anomaly, chronic granulomatous disease of childhood, severe combined immunodeficiency, and ataxia-telangiectasia)
3. Nonneoplastic RBC abnormalities: four patients with secondary polycythemia, one patient with malignancy-associated anemia, one patient with autoimmune hemolytic anemia, one patient with iron deficiency anemia, and one patient with thalassemia major.

Neoplastic lymphoproliferative disorders (LPD) and plasma cell dyscrasias (PCD). This group of patients consisted of five patients with acute lymphoblastic leukemia, nine patients with chronic lymphocytic leukemia, two patients with the Sézary syndrome, one patient in the leukemic phase of lymphoma, two patients with Burkitt's lymphoma, two patients with nodular sclerosing Hodgkin's

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disease, one patient with plasma cell leukemia, and three patients with plasma cell myeloma.

Neoplastic MPD The following three types of MPD were included: dysmyelopoietic syndrome (DMPS),[35] eight patients; acute nonlymphoblastic leukemias (French-American-British classification M1 to M6),[36] 25 patients; and chronic MPD, nine patients with chronic myelogenous leukemia (CML), five patients with primary thrombocythemia (PT), one patient with megakaryocytic myelosis, three patients with agnogenic myeloid metaplasia with myelofibrosis, and three patients with polycythemia vera.

Preparation of Buffy Coats for Electron Microscopy

Blood samples were collected by venipuncture using EDTA as an anticoagulant. All healthy volunteers and patients were advised of this procedure and its attendant risks in accordance with our institutional guidelines and gave informed consent. A buffy coat plug was obtained using the centrifugation and in situ fixation procedures originally described by Anderson.[37] The blood was centrifuged in Wintrobe tubes[38] at 2,500 rpm for ten minutes in a Sorvall GLC-1 swinging bucket clinical centrifuge. The plasma was removed and the buffy coat plug fixed for two hours in 3% glutaraldehyde (buffered with 0.1 mol/L phosphate buffer, pH 7.2). The buffy coats were removed and sliced perpendicularly to the top surface of the plug before postfixation in 1% osmium tetroxide as previously described.[34] Platelets prepared in this manner instead of first obtaining a platelet-rich plasma (PRP) fraction allowed for the examination of both heavy (near-white cell layer) and light platelets. The buffy coats were dehydrated in a graded series of ethanols and embedded in Spurr's low-viscosity epoxy resin [39] One-micron sections were cut and stained with toluidine blue O. Selected blocks showing a good cross section through the buffy coat cell layers were thin-sectioned with a diamond knife on a MT-5000 ultramicrotome (DuPont/Sorvall, Claremont, Calif). The sections were mounted on uncoated 200-mesh copper grids, stained with lead citrate and 5% uranyl acetate (made up in 50% methanol), and examined under an HU-12 electron microscope (Hitachi, Mountain View, Calif).

Morphometric Procedures

Each grid square of the platelet layer was first thoroughly examined at a scope magnification of 7,000× without the use of the attached 10× ocular system. Large, irregularly shaped or fusion-type granule profiles could then be easily detected when compared to an extensive array of smaller and/or less irregularly shaped granules at a relatively low magnification. The profiles of approximately 1,000 different platelets were examined under the electron microscope in each case. Usually only one epoxy block had to be cut from each patient with a normal or elevated platelet count to examine 1,000 different platelets since a single ultrathin section almost covers the entire grid surface. Each grid square, therefore, contains the profiles of different platelets and not serial sections of the same platelets. In those cases with low platelet counts, as many as four epoxy blocks were cut and analyzed. Any large or bizarre granule profiles on inspection were photographed at a scope magnification of 10,000× to 27,000× The number of granule profiles photographed per case varied from one (some normal subjects) to over 40 (diseased groups) depending upon the frequency of abnormal-appearing granule profiles present. For this study we concentrated specifically on finding giant and fusion granule profiles, which were rare in all of the normal subjects and few in number in most of the control cases. The very narrow and elongated rod-shaped granules frequently seen in normal platelets were not photographed.

The negatives were printed on 8 × 10-in sheets of Kodabromide paper and morphometrically analyzed using a MOP-3 computerized image analyzer[40] (Carl Zeiss, Inc, NY). The granule membrane was traced in all cases. In oblique sections in which a portion of the membrane became less distinct, the granule contents were outlined and the resultant tracing made continuous with the more distinct adjacent membrane profiles. The perimeter (P), area (A), and form factor (FF) values were then computed for each granule photographed. FF is a formula that is programmed into our MOP-3 image analyzer[41] and gives a measure of contour irregularity.[42] The degree of irregularity of a given profile is expressed by its FF value, which is obtained by inserting the area and perimeter of the contour into the formula. $FF = 4\pi A/P^2$ If πr^2 is substituted for A and $2\pi r$ is substituted for P, where r is the radius, all terms cancel out and the FF value for a circle becomes 1.0. The degree of contour irregularity increases as the FF value decreases. A granule contour with a FF value of 0.20 would therefore have a shape that is only 20% of a circle.

Statistical Analysis

The mean megagranule group value and SEM for each morphometric parameter was determined by first obtaining the largest perimeter and area and lowest form factor values for all of the unusual granule profiles photographed and analyzed from each patient in each clinical group and then averaging these values within each group. The mean group morphometric values were statistically compared using Student's *t* test. The difference between two means was considered statistically significant if the *P* value was less than .05

RESULTS

Ultrastructure of Normal Platelets

The normal human platelet (Fig 1A) is ultrastructurally complex and contains mitochondria, a dense tubular system, an open canalicular system, dense bodies, α-granules, microfilaments, microtubules, and glycogen. It is generally believed that platelet organelles with eccentric nucleoids are α-granules (Fig 1A). Some granules without nucleoids could also be α-granules in which the plane of the section does not include the nucleoid. α-granule profiles that include the nucleoid in the plane of the section are variable in size and shape but are usually small (mean diameter of 0.23 μ with a range of 0.15 to 0.32 μ) and round or oval. Occasionally, giant α-granule profiles are observed that also appear round in contour (Fig 1B). The electron density of the α-granule matrix substance is appreciably less, however, than that of the dense granule (Fig 1A). During electron microscopic examination of the profiles of approximately 30,000 different platelets from 30 normal subjects, occasional fused (Figs 2A and 2B) and irregularly shaped granule profiles (Fig 2B) were found. Some of these fusion-type granule profiles appeared qualitatively similar to the fusion granule profiles reported in patients with preleukemia and MML.[15]

Ultrastructure of Platelet Giant and Fusion Granules from Patients with Neoplastic MPD

A typical fusion granule profile commonly found in patients within the neoplastic MPD group is highly irregular in shape, has appreciable electron density, and consists of the contents of numerous α-granules (Fig 3A). The number of α-granules that fuse to form the giant granules is quite variable. In some profiles, as many as 20 individual granules could be identified within one megagranule profile (Fig 3B).

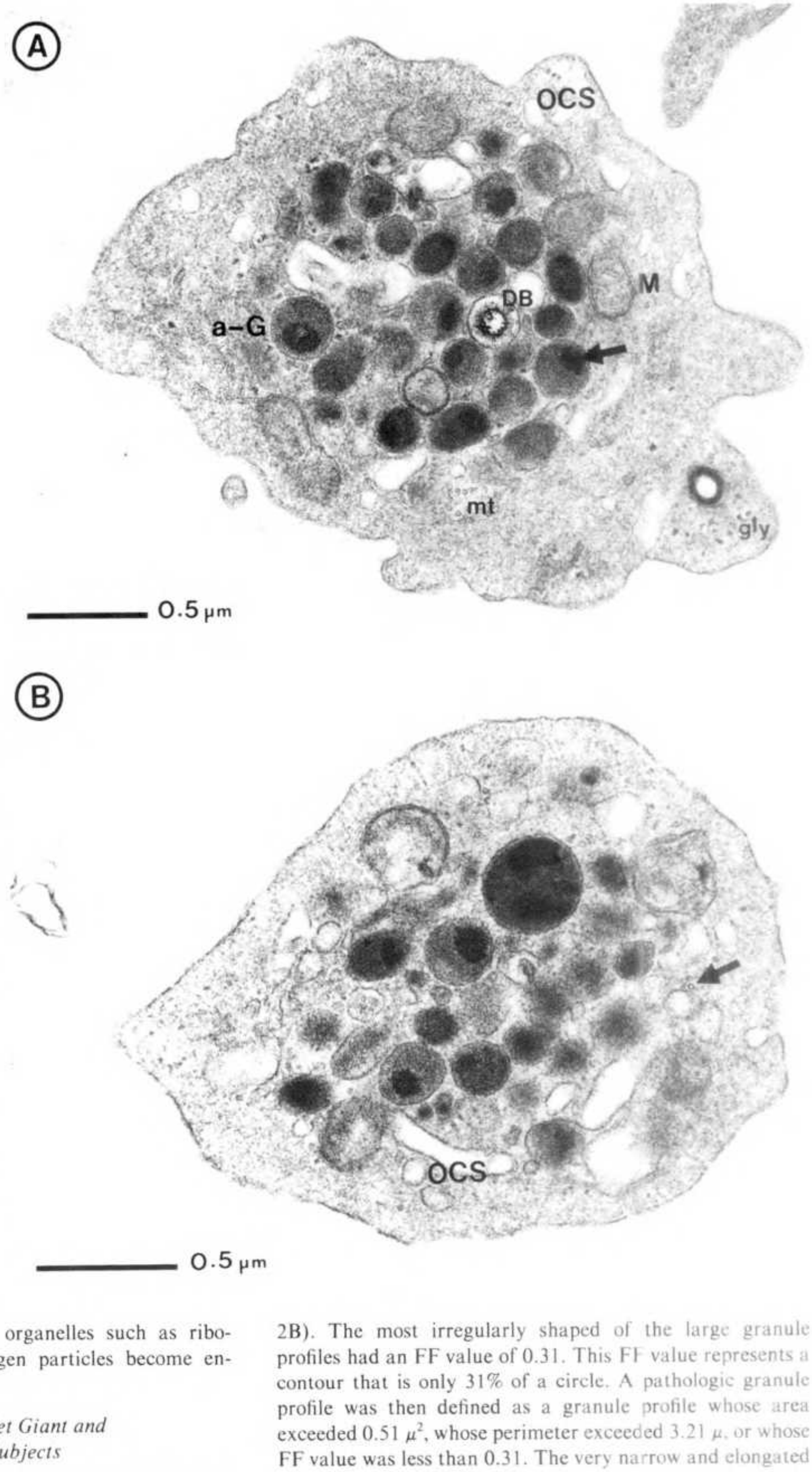

Fig 1. Electron micrographs of platelet profiles obtained from normal subjects (uranyl acetate, lead citrate). (A) The α-granules (a-G) contain eccentric nucleoids (arrow) and appear round to oval in shape with some profiles slightly larger than mitochondria (M). The dense body (DB) is more electron dense than the α-granules. Also shown are glycogen particles (gly), the cross sections of microtubules (mt), and elements of the open canalicular system (OCS) (original magnification × 46,400; current magnification × 34,800). (B) This platelet profile contains a giant granule that is round in contour and contains two eccentric nucleoids. Also shown is a microtubular cross section (arrow) and elements of the open canalicular system (original magnification × 54,800; current magnification × 41,100).

Occasionally, other cytoplasmic organelles such as ribosomes, mitochondria, and glycogen particles become entrapped within the megagranule.

Morphometric Analysis of Platelet Giant and Fusion Granules From Normal Subjects

The largest granule profile observed in all 30 control subjects had an area of 0.51 μ^2 and a perimeter of 3.21 μ (Fig 2B). The most irregularly shaped of the large granule profiles had an FF value of 0.31. This FF value represents a contour that is only 31% of a circle. A pathologic granule profile was then defined as a granule profile whose area exceeded 0.51 μ^2, whose perimeter exceeded 3.21 μ, or whose FF value was less than 0.31. The very narrow and elongated rod-shaped granules frequently seen in normal platelets were not included in this study.

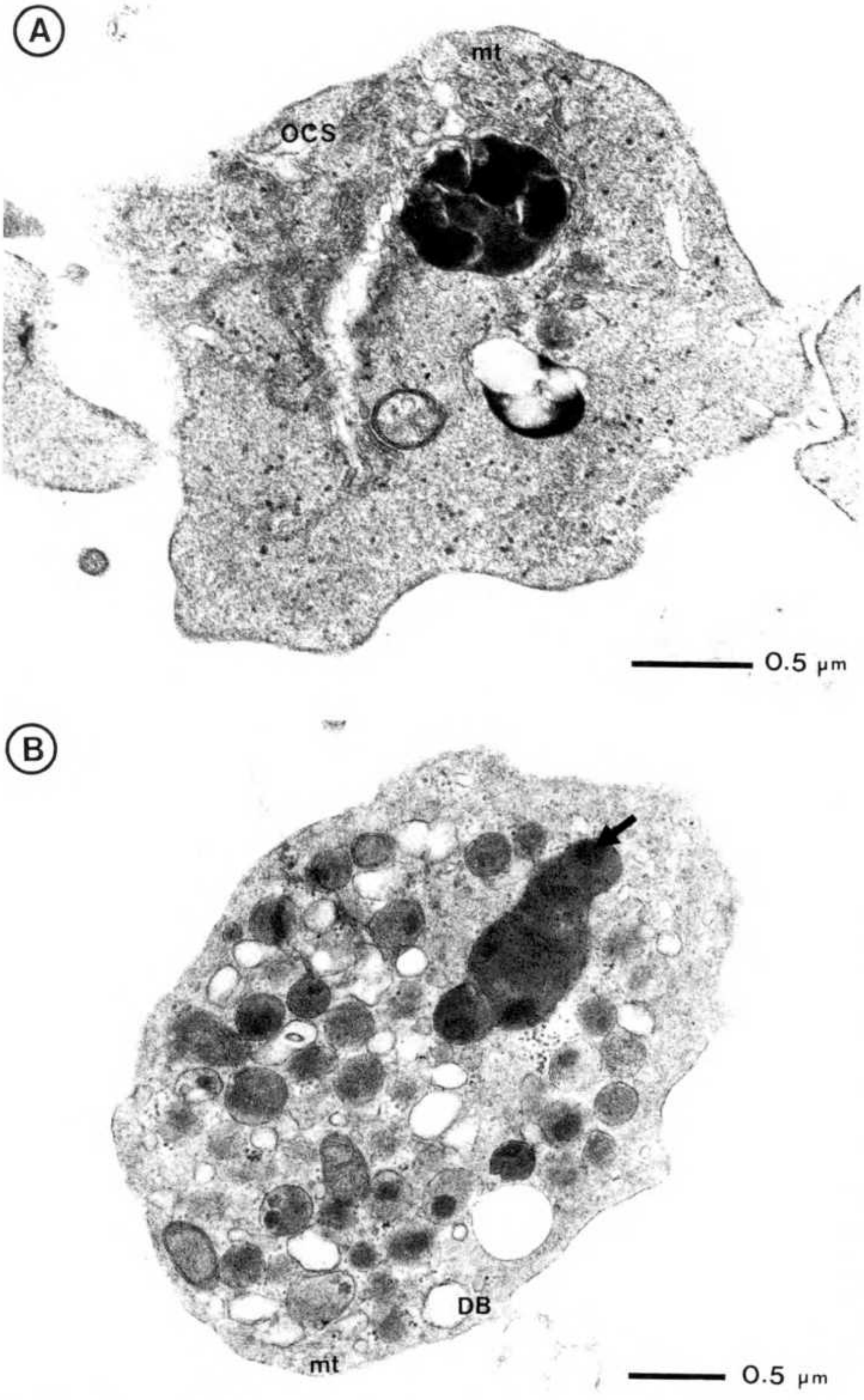

Fig 2. Electron micrographs of platelet profiles obtained from normal subjects (uranyl acetate, lead citrate). (A) This platelet profile contains a type of fusion granule with a slightly oval shape. The profiles of seven granules can be seen within the fusion granule. Also shown are elements of the open canalicular system, scattered glycogen particles, and the cross section of a microtubule (original magnification × 43,700; current magnification × 32,775). (B) This platelet profile contains a fusion-type granule with an irregular contour. Several distinct α-granule nucleoids (arrow) can be seen within the fusion granule. A dense body containing a sparse amount of electron-dense material and microtubule profiles is also shown (original magnification × 35,200; current magnification × 26,400).

Morphometric and Ultrastructural Analysis of Platelet Giant and Fusion Granules in Diseased Control Subjects With No Evidence of an Underlying Neoplastic MPD

Twenty percent of these diseased control subjects had some abnormal platelet granule profiles using the morphometric criteria outlined above (Table 1). The number, however, of morphometrically defined abnormal platelet granule profiles was low in these clinical groups. Only 57 granule profiles were determined to be abnormal after image analysis. Overall, abnormal granule profiles were identified in approximately 0.04% of the platelet profiles from these patients.

Morphometrically defined abnormal granule profiles were

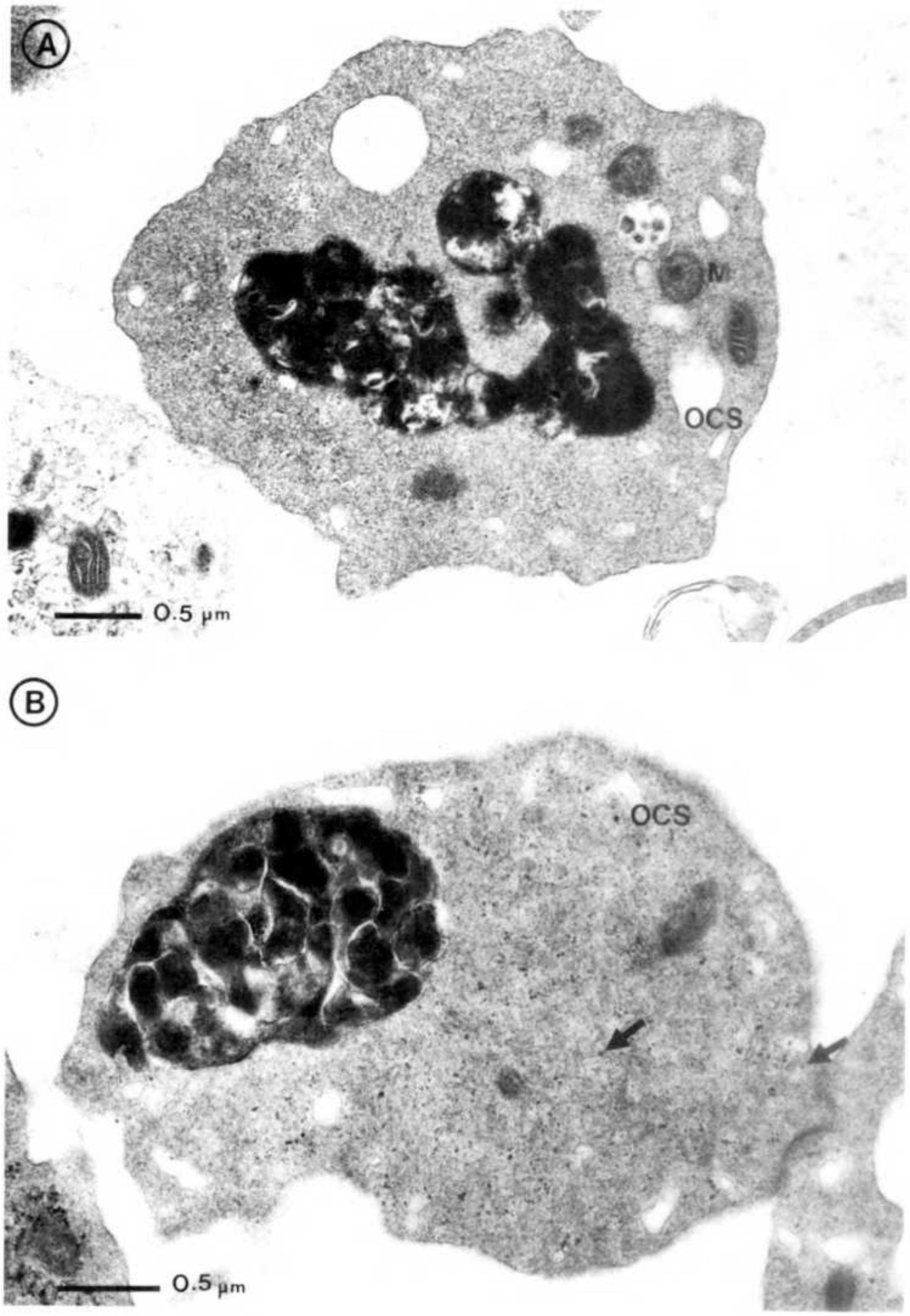

Fig 3. Electron micrographs of platelet profiles obtained from patients with neoplastic MPD (uranyl acetate, lead citrate). (A) Platelet profile from a patient with DMPS. The giant fusion granule shown here has a heterogeneous electron-dense content and a highly irregular contour. This abnormal granule had the largest perimeter and area of the entire neoplastic MPD group. Also shown are mitochondria and elements of the open canalicular system (original magnification × 33,800; current magnification × 25,350). (B) Platelet profile from a patient with erythroleukemia. The giant fusion granule shown here contains the outlines of at least 20 distinct granules. Glycogen particles also form part of the contents of this megagranule. Scattered microtubular cross sections (arrows) and elements of the open canalicular system are present (original magnification × 39,900; current magnification × 29,925). Figure 3B is reprinted with permission from Proc EMSA, pp 786–787, 1983 (San Francisco Press, Box 6800, San Francisco).

identified in at least one patient in each of the following control groups: steroid-treated normal subjects, nonneoplastic platelet disorders, nonneoplastic WBC disorders, nonneoplastic RBC disorders, and neoplastic LPD and PCD. Scatter plots showing the distribution of the most abnormal granule profiles obtained from each patient in each of these control groups are shown in Figs 4 and 5. The most abnormal granule profiles were defined as the granule profile with either the largest perimeter, the largest area, or the lowest form factor.

Typical giant fusion granule profiles that were indistinguishable from those found in the neoplastic MPD groups were prominent in patients with some congenital platelet disorders such as the May-Hegglin anomaly and the Bernard-Soulier syndrome (Fig 6A).

Patients with reactive thrombocytosis as a group could be distinguished from normal subjects on the basis of the platelet granule perimeter ($P < .001$) and area ($P < .001$) (Table 2). Mean form factor values had no discriminatory value ($P > .1$) (Table 2).

Table 1. Incidence of Abnormal Platelet Granules in Control Groups

Clinical Group	No. Cases Studied	No. Cases With Abnormal Granule Profiles	No. Granule Profiles Morphometrically Analyzed
Normal subjects	30	—	255
Normal subjects, steroid treated	12	1	152
Nonneoplastic platelet disorders	54	14	713
Nonneoplastic WBC disorders	26	2	267
Nonneoplastic RBC disorders	8	3	118
Neoplastic LPD and PCD	27	5	328
Total	157	25	1,833

Nineteen percent of the patients with neoplastic LPD/PCD had abnormal granule profiles (Table 1, Figs 4 and 5). The neoplastic LPD/PCD group could be distinguished from normal subjects using all three morphometric parameters (perimeter, $P < .01$; area, $P < .02$; FF, $P < .02$) (Table 2).

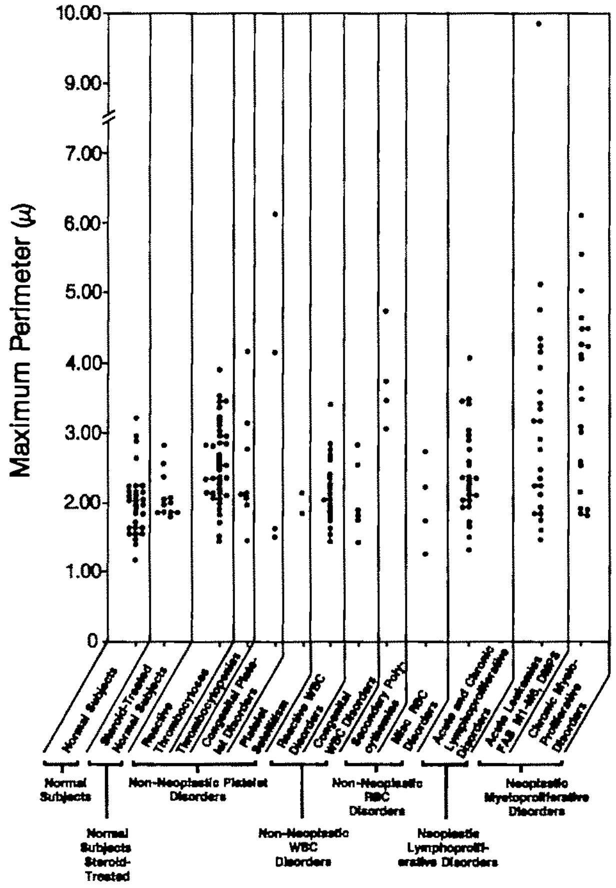

Fig 4. Scatter plot showing the distribution of platelet granule profiles with the largest perimeters morphometrically identified in each of the clinical groups. Each point represents a separate individual. The neoplastic LPD also include the plasma cell dyscrasias.

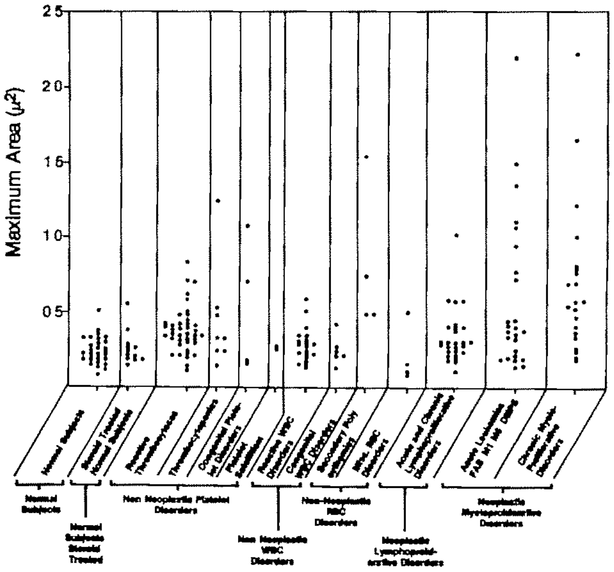

Fig 5. Scatter plot showing the distribution of platelet granule profiles with the largest area morphometrically identified in each of the clinical groups. Each point represents a separate individual. The neoplastic LPD also include the plasma cell dyscrasias.

Three out of the 4 patients with secondary polycythemia had abnormal granule profiles (Figs 4 and 5). One patient had a megagranule profile (Fig 6B) that was three times the size of the largest granule profile (Fig 2B) obtained from normal subjects.

Although one subject out of 12 in our steroid-treated control group had a single granule profile that was slightly larger (0.55 μ^2) than the largest granule profile (0.51 μ^2) from untreated normal subjects, there was no statistical difference between the steroid-treated group as a whole and normal subjects using all three morphometric parameters.

Morphometric Comparison of Platelet Giant and Fusion Granules Between Patients With Neoplastic MPD and All Other Groups

Fifty-seven percent of the patients with neoplastic MPD were determined to have abnormal platelet granule profiles (Table 3). The total number of morphometrically defined abnormal platelet granule profiles was five times more numerous in the neoplastic MPD group compared to the diseased control group. One hundred four granule profiles were determined to be abnormal after image analysis. Overall, abnormal granule profiles were identified in approximately 0.2% of the platelet profiles examined.

The morphometric parameters that were most helpful in discriminating between patients with neoplastic MPD and all other normal and diseased individuals as a group were perimeter ($P < .001$) and area ($P < .001$) (Table 2). The difference between the mean FF values calculated from the neoplastic MPD and control groups was not statistically significant ($P > .05$) (Table 2). Scatter plots showing the distribution of the most abnormal granule profiles obtained from each patient in the neoplastic MPD groups are shown in Figs 4 and 5.

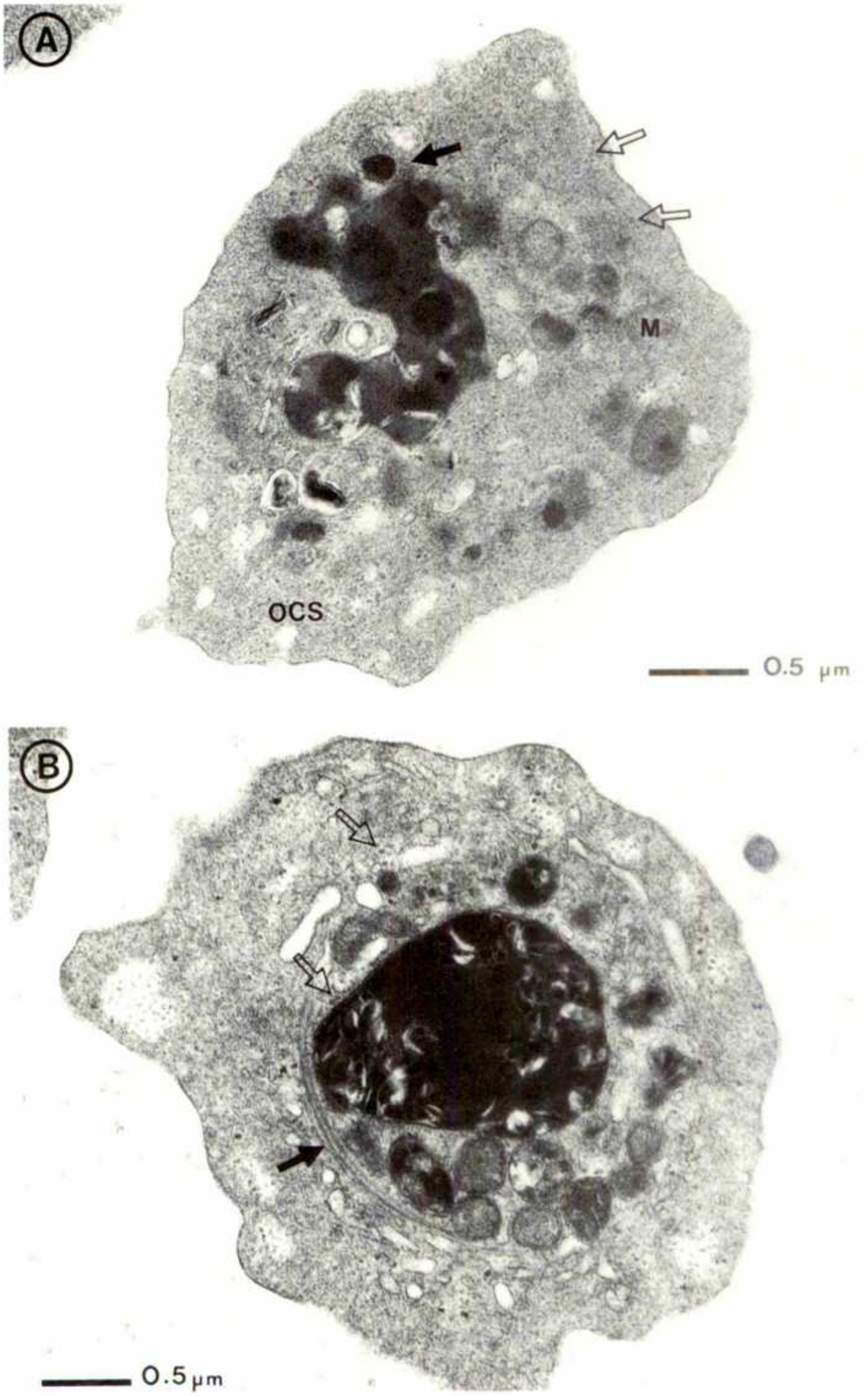

Fig 6. Electron micrographs of platelet profiles from control diseased patients without any clinical evidence of a neoplastic MPD (uranyl acetate, lead citrate). (A) Platelet profile from a patient with the Bernard-Soulier syndrome. This platelet profile contains a giant fusion granule with a highly irregular contour. One α-granule (solid arrow) is seen touching the membrane of the fusion granule. Mitochondria, elements of the open canalicular system, and scattered microtubular cross sections (open arrows) are present (original magnification × 35,200; current magnification × 26,400). (B) Platelet profile from a patient with polycythemia secondary to chronic obstructive pulmonary disease. This platelet appears activated as evidenced by the centripetal location of organelles and the surrounding band of microtubules (solid arrow). Scattered microtubular cross sections are also seen (open arrows) (original magnification × 31,900; current magnification × 23,925). Figure 6B is reprinted with permission from Proc EMSA, pp 786–787, 1983 (San Francisco Press, Box 6800, San Francisco).

Platelet granule perimeter ($P < .001$) and area ($P < .001$) distinguished the neoplastic MPD group from the individual clinical groups associated with nonneoplastic cellular proliferations such as granulocytic leukemoid reactions, eosinophilia, monocytosis, and the neoplastic LPD/PCD group (Table 2). Mean granule FF values had no discriminatory value ($P > .5$).

Patients with neoplastic chronic MPD, most of whom are associated with high platelet counts, could be distinguished as a group from the reactive thrombocytosis group on the basis of all three morphometric parameters: perimeter, $P < .001$; area, $P < .01$; FF, $P < .01$ (Table 2). The large granule profiles photographed from the neoplastic chronic MPD group were twice as large as the large granule profiles from the reactive thrombocytosis group and were more irregular in contour (Table 2). When patients with reactive thrombocytosis were compared as a group to patients with PT (a neoplastic disorder) as a group, only granule perimeter

Table 2. Morphometric Comparison of Platelet Giant and Fusion Granules Between Clinical Groups

Morphometric Parameter	Clinical Groups (Mean Group Values ± SEM)*									
	Normal Subjects	All Patients Without a Neoplastic MPD†	Reactive Thrombocytosis	Reactive Granulocytosis/Monocytosis‡	Neoplastic LPD/PCD Group	All Neoplastic MPD Groups	Chronic Neoplastic MPD Group	PT	Acute Non-Lymphoblastic Leukemias	DMPS
Perimeter (μ)	2.00 ± 0.09	2.43 ± 0.07	2.64 ± 0.09	2.32 ± 0.14	2.44 ± 0.13	3.38 ± 0.23	4.05 ± 0.31	3.76 ± 0.62	2.64 ± 0.25	4.47 ± 0.88
Area (μ^2)	0.24 ± 0.02	0.35 ± 0.02	0.38 ± 0.02	0.30 ± 0.03	0.34 ± 0.04	0.64 ± 0.06	0.79 ± 0.16	0.89 ± 0.38	0.39 ± 0.09	1.00 ± 0.22
FF	0.63 ± 0.03	0.58 ± 0.01	0.57 ± 0.03	0.58 ± 0.05	0.52 ± 0.03	0.54 ± 0.02	0.46 ± 0.03	0.47 ± 0.07	0.58 ± 0.04	0.52 ± 0.07
n	30	127	40	13	27	46	12	5	17	8

*See Statistical Analysis section of Materials and Methods for details.

†Includes all control clinical groups without neoplastic MPD excluding normal subjects.

‡Includes six patients with a granulocytic leukemoid reaction, five patients with eosinophilia, and two patients with monocytosis.

had discriminatory value ($P < .05$) (Table 2). On an individual basis, a patient could be diagnosed as having PT only if the perimeter of the platelet granules exceeded the highest perimeter value in the reactive thrombocytosis group. No morphometric parameter was useful, however, in distinguishing patients with PT as a group from patients with other types of neoplastic chronic MPD (Table 2).

Patients with acute nonlymphoblastic leukemia as a group could be distinguished from patients with DMPS using the mean granule area only ($P < .05$) (Table 2). The differences in mean granule perimeter ($P > .05$) and FF ($P > .2$) values were not statistically significant. Twenty-five percent of the patients with DMPS had no morphometrically defined megagranule profiles, and 12% of the patients with acute nonlymphoblastic leukemia had megagranule profiles. The DMPS group had the largest mean megagranule profile area (1.00 μ^2) of all clinical groups; these megagranule profiles were four times larger than the largest granule profiles from normal subjects (Table 2). The most abnormal granule profile in the study was also obtained from a patient with DMPS (Fig 3A). The perimeter was 9.8 μ, the area was 2.2 μ^2, and the shape was only 23% of a circle (FF = 0.23).

Table 3. Incidence of Abnormal Platelet Granules in Neoplastic MPD

Neoplastic MPD	No Studied	No Cases With Abnormal Granule Profiles	No Granule Profiles Morphometrically Analyzed
Acute nonlymphoblastic leukemias			
AML	7	3	80
Acute MML	3	2	14
Acute progranulocytic leukemia	3	0	18
Acute monoblastic leukemia	1	0	5
Acute basophilic leukemia	1	0	3
Erythroleukemia	2	1	8
DMPS	8	6	162
Chronic MPD			
CML	9	3	67
PT	5	5	84
Megakaryocytic myelosis	1	0	10
Agnogenic myeloid metaplasia with myelofibrosis	3	3	28
Polycythemia vera	3	3	79
Total	46	26	558

DISCUSSION

In this study we attempted to remove the subjectivity in the identification of abnormal platelet granule profiles by using computerized image analysis techniques. Occasional atypical-appearing granule profiles found in normal subjects can be confused, on a subjective basis, with some of the fusion granule profiles that have been described in patients with preleukemia[13–17,30] and MML.[3,13–17,30] We have morphometrically analyzed over 2,000 atypical granules from normal subjects, patients with 13 different neoplastic MPD, and other diseased control subjects that included patients with a variety of reactive and congenital WBC, RBC, and platelet disorders. We have determined, after examining the platelets of 30 normal subjects, that one could define an abnormal granule profile as one whose perimeter exceeded 3.21 μ, whose area exceeded 0.51 μ^2, or whose shape was less than 31% of a circle. Maldonado[14] has reported that some giant granules found in normal humans can have a diameter of 0.8 μ. This would correspond to an area of 0.50 μ^2 (assuming a circular profile) and is very close to our measured value of 0.51 μ^2.

Although platelet giant fusion granules were initially reported to be possible cell markers for preleukemia and MML,[15,17] we have shown using image analysis that they are non–disease-specific and can be identified in 20% of the diseased control patients who have no clinical evidence of an underlying neoplastic MPD. Approximately three times as many patients with neoplastic MPD had morphometrically defined abnormal granule profiles compared with other diseased control subjects. The morphometric parameters that had the greatest discriminatory value were perimeter and area. Since the neoplastic MPD group could be distinguished from the neoplastic LPD/PCD group using these parameters, platelet analysis may have some significance in the diagnosis of poorly differentiated leukemias. Distinguishing PT from secondary thrombocytosis can also be a diagnostic problem. We have morphometrically determined that patients with PT as a group could be distinguished from patients with secondary thrombocytosis as a group when the mean group maximum perimeter values are compared. When diagnosing an individual patient, however, the maximum megagranule perimeter for that patient must exceed the maximum individual value in the secondary thrombocytosis group for the platelet proliferation process to be suggestive of a neoplastic process. Megagranule morphometric analysis, therefore, has specificity but may lack sensitivity.

Ultrastructural studies from other laboratories have indicated that on a subjective basis giant-appearing granule profiles could be found in neoplastic MPD other than preleukemia and MML. These included acute myeloblastic leukemia (AML),[30] acute monocytic leukemia (AMoL),[30] CML,[29,30] PT,[20,21,27,28,31-33] and primary myelofibrosis (MF).[18] In addition to preleukemia and MML, we have morphometrically confirmed the presence of abnormal giant fusion granules in AML, CML, PT, and MF. We could not morphometrically identify any abnormal granule profiles in our case of AMoL. As a result of this study, we can now add erythroleukemia and polycythemia vera to the list of neoplastic MPD that are known to contain these megagranules. McClure et al[43] described by light microscopy many large platelets with heavy granules in a 5-year-old boy with chronic erythroleukemia. These heavy granules probably correspond to the giant fusion granules that we observed in one of our two cases of erythroleukemia at the ultrastructural level (see Fig 3B).

Platelet giant fusion granules have been previously reported in some congenital WBC disorders.[22-25] We have morphometrically confirmed the presence of giant fusion granule profiles in one of our two patients with the May-Hegglin anomaly. Giant platelet granules have not been reported in all cases of the CHS. White[23] and Parmley et al[24] illustrate giant-appearing granule profiles in the platelets of their patients with the CHS. Davis and Douglas,[44] on the other hand, state that the only bone marrow elements that lack giant granules are the megakaryocytes and platelets. Enlarged platelet α-granule profiles were also not identified in the bovine,[44,45] mink,[44,46] and mouse[47] homologues of the human Chédiak-Higashi trait. We have not been able to morphometrically identify any large granule profiles in our patient with the CHS.

We have also morphometrically identified for the first time giant fusion granules in the Bernard-Soulier syndrome, which, like the May-Hegglin anomaly, is characterized by the presence of giant platelets. These megagranule profiles were easy to find and were indistinguishable ultrastructurally from the giant fusion granule profiles found in the neoplastic MPD groups. Previous ultrastructural studies on patients with the Bernard-Soulier syndrome did not report the presence of any megagranule profiles.[48-50] Our case was complicated, however, by the fact that our patient was splenectomized 26 years prior to our ultrastructural evaluation. Although the pitting role that the spleen plays in the removal of Howell-Jolly bodies, autophagic vacuoles, Heinz bodies, and siderotic granules[51-54] from circulating erythrocytes has been well documented, the role that the spleen plays in the pitting of megagranules from platelets needs to be evaluated.

Among the benign RBC disorders, morphometrically defined megagranule profiles were observed in three of the four patients with secondary polycythemia. The polycythemia in these three patients was secondary to hypoxemia. Two of the patients had chronic obstructive pulmonary disease, and one patient had the pickwickian syndrome. The high incidence of megagranules in this clinical group has not been previously reported.

Although platelet giant fusion granules may be likened to leukocyte Auer rod formation or the pseudo-CHA of leukemia,[55] we have shown in this morphometric study that they are found in many different nonneoplastic conditions. It is therefore possible that the pathogenesis of these megagranules may be similar in both the nonneoplastic and neoplastic conditions. On the other hand, the many different clinical conditions that exist in the diverse patient population studied makes it equally plausible that the formation of giant and fusion granules may have multiple causes.

Our studies suggest that an oxygen-poor microenvironment may be conducive to α-granule fusion with subsequent megagranule formation. Three patients with secondary polycythemia had low pO_2 blood levels, and all had platelet giant granules. Support for a hypoxic cause may come from platelet storage experiments. White and Clawson[56] showed that giant granules can occur in normal platelets during prolonged storage. These stored platelets may be exposed to hypoxic conditions. The present study has also shown that morphometrically defined abnormal platelet granule profiles were five times more numerous in the neoplastic MPD group compared to the diseased control group. Thus, additional factors such as abnormal cellular regulatory mechanisms common to the neoplastic state, abnormal granule formation, and changes in the cellular microenvironment may also contribute to megagranule formation.

As a result of this study we have established ultrastructural morphometric criteria that can be used to define the pathologic nature of platelet giant and fusion granule profiles. We have determined, with the use of morphometry, that giant fusion granules are non–disease-specific and can be seen in patients who have no evidence of an underlying neoplastic MPD. Giant fusion granules are, however, more prevalent in the neoplastic MPD group, but because of their nonspecificity, may only have diagnostic significance for the individual patient in specific clinical settings. The formation of megagranules may have multiple pathogenic mechanisms. Factors such as hypoxia, splenectomy, hypersplenism, abnormal cellular regulatory mechanisms, and abnormal thrombopoiesis may play a role in their formation. The mechanism of granule fusion remains to be investigated.

ACKNOWLEDGMENT

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Identification of Glycoprotein Ib as a Target for Autoantibody in Idiopathic (Autoimmune) Thrombocytopenic Purpura

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An antibody (DIL) from a patient with idiopathic thrombocytopenic purpura (ITP) was shown to have autospecificity on the basis of reactions with autologous platelets that were identical to those obtained with platelets from normal subjects. DIL antibody also reacted strongly in an immunofluorescence test with platelets from a patient with Glanzmann's thrombasthenia, but failed to react with platelets from a patient with the Bernard-Soulier syndrome who was known to be deficient in glycoprotein Ib (GPIb). Purified GPIb and control platelets, but not Bernard-Soulier platelets, inhibited the lytic activity of DIL. Using the GPIb-specific monoclonal antibody AP1 and one-dimensional rocket electrophoresis into gels containing rabbit antihuman platelet membrane antibody, it was shown that staphylococcal protein A-Sepharose beads coated with DIL antibody selectively remove GPIb from solubilized platelet preparations. By crossed immunoelectrophoresis it was found that DIL recognizes a determinant on GPIb on the membrane side of the cleavage site of the platelet calcium-activated protease (calpain). These studies provide direct evidence for binding of a platelet autoantibody to a determinant on GPIb relatively close to the site of insertion of this protein into the platelet membrane.

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IDIOPATHIC thrombocytopenic purpura (ITP) is a common hemorrhagic syndrome characterized by a reduced number of circulating platelets, normal to increased numbers of megakaryocytes, and accelerated platelet destruction.[1-3] Abundant evidence has been obtained to support the view that, at least in adults, destruction of platelets in ITP is mediated by platelet-specific autoantibodies. Recent studies have begun to characterize the platelet membrane constituents recognized by autoantibodies in this disorder. Several reports have indicated that a high percentage of ITP autoantibodies recognize determinants on platelet membrane glycoproteins IIb and/or IIIa (GPIIb-IIIa).[4-6] In a recent study, Woods et al, using a system in which GPIb was immobilized in microtiter wells by the monoclonal antibody AP1, reported that plasma from three of 106 patients with ITP caused IgG to be deposited in the wells containing GPIb.[7] Antibodies from two of the three patients precipitated a protein with a molecular weight consistent with that of GPIb from radioiodinated platelet extracts, providing presumptive evidence that some autoantibodies in ITP recognize determinant(s) on the GPIb molecule.

We recently had an opportunity to study a patient with ITP whose plasma contained a complement-fixing autoantibody that was available in large quantities as a result of treatment by exchange transfusion.[8] Described here are studies providing direct evidence that this autoantibody reacts with a site on platelet membrane GPIb.

METHODS

Platelet aggregation. Aggregations were performed in a Bio-Data platelet aggregation profiler model PAP-4 (BioData Corp, Hatboro, Pa). A quantity of 200 μL of citrated platelet-rich plasma (PRP) at 430,000 platelets/μL was incubated for five minutes at 37 °C with 200 μL of either autologous or antibody-containing platelet-poor plasma (PPP). Fifty microliters of monoclonal AP1 or AP2 antibody (1 mg/mL) was added at this time. Aggregation was initiated by the addition of 20 μL of ristocetin (33.3 mg/mL), 2×10^{-4} mol/L adenosine diphosphate, or 5.5×10^{-4} mol/L epinephrine to a final concentration of 1.57 mg/mL, 9.5×10^{-6} mol/L, and 2.6×10^{-5} mol/L respectively. The reaction was allowed to continue for at least five minutes.

Isolation of Bernard-Soulier syndrome platelets Bernard-Soulier syndrome (BSS) platelets were isolated from whole blood by an adaptation of the method of Solum et al[9] as described by Nurden et al.[10] One volume of acid citrate dextrose anticoagulant (ACD) was added to nine volumes of whole blood from a BSS patient and a normal control. Prostaglandin E_1 (PGE_1) was added to a final concentration of 20 ng/mL. Four milliliters of whole blood were carefully layered over 6 mL of 10.9% sodium metrizoate and 2.6% dextran. The test tube racks were tilted and remained at room temperature for one hour. The red cells sedimented through the dextran, and the PRP was collected and pooled. The remaining red cells in the PRP were allowed to settle by gravity, and the secondary PRP was aspirated and counted. These cells were used in immunofluorescence and ^{51}Cr release inhibition assays.

Platelet suspension immunofluorescence test. The PSIFT was performed as previously described by von dem Borne et al[11] with the modifications of Kunicki and Aster.[12]

Inhibition of antibody-mediated lysis of ^{51}Cr-labeled platelets. Inhibition of antibody-mediated lysis of ^{51}Cr-labeled platelets was performed by a modification of a previously described method.[12] Twenty microliters of platelets or purified GPIb were mixed with 50 μL of antiserum and incubated at room temperature for 30 minutes. Then, 2×10^6 ^{51}Cr-labeled papain-treated platelets, 0.1 mL normal human plasma anticoagulated with EDTA (8 mmol/L final concentration) as a source of complement, and 20 μL 0.1 mol/L $MgCl_2$ were added and incubated for two hours at 37 °C. The mixture was then diluted with 2 mL of a mixture consisting of 13.4 mmol/L EDTA per 145 mmol/L NaCl and centrifuged, with ^{51}Cr released in the supernatant measured in a gamma scintillation counter. The amount of ^{51}Cr released is inversely related to the amount of inhibition by platelets or GPIb or antibody-mediated lysis of target cells.

Absorption of solubilized platelets by antibody-coated staphylo-

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coccal protein A–Sepharose. An eluate containing DIL autoantibody was obtained by absorbing 300 mL of DIL plasma with 3 × 10^{10} washed platelets at room temperature for 30 minutes. After three washes in .1 mol/L phosphate-buffered saline (PBS) with a pH of 7.4, the autoantibody was eluted from the platelets at pH 3.0, neutralized, and stored at −80 °C.[13] The amount of IgG in the eluate was determined by electroimmunoassay.[14] About one third of the IgG in this preparation represents platelet-reactive antibody as determined by electroimmunoassay before and after adsorption by platelets. A quantity of 100 μg of DIL IgG or control IgG was incubated with 20 μL of washed staphylococcal protein A–Sepharose (protein A–Sepharose CL-4B; Pharmacia Fine Chemicals, Piscataway, NJ) at room temperature with rotation for 30 minutes. The beads were washed three times in PBS buffer, pH 7.0, and aspirated to dryness. Washed, type 0 control platelets were solubilized in 1% Triton X-100 (BioRad, Richmond, Calif.), with or without leupeptin (200 μg/10^9 platelets), in 0.1 mol/L tris (hydroxymethyl) aminomethane glycine buffer, pH 8.75, by vortexing at 4 °C for 30 minutes. Solubilized platelets were ultracentrifuged at 35,000 g for 30 minutes to remove nonsolubilized cytoskeletal components. The amount of protein in the soluble portion was determined by the method of Markwell et al.[15] Solubilized platelets were preadsorbed with human albumin (SPA)-Sepharose to remove proteins reacting nonspecifically with this reagent. Fifty micrograms of preadsorbed solubilized platelet protein were then added to the antibody-coated beads and rotated at room temperature for 30 minutes. The mixture was centrifuged at 1500 g for five minutes.

The supernatants were then analyzed by quantitative electroimmunoassay[16] in 1% agarose gel containing rabbit antihuman platelet membrane antibody and radioiodinated monoclonal antibody specific for either GPIb (AP1) or GPIIb-IIIa (AP2). Characteristics of these antibodies have been described previously.[17] In this system, the nonprecipitating monoclonal antibody binds to membrane glycoprotein precipitated by the rabbit antibody, permitting individual precipitates to be identified by autoradiography. The supernatants were also analyzed by crossed immunoelectrophoresis[18] using the same principle. The final preparations were stained with Coomassie blue R, dried, and autoradiographed.

Source of autoantibody DIL. Mr DIL is a 76-year-old man who presented with profound thrombocytopenia and life-threatening symptoms in Oct 1981. His serum contained a potent, complement-fixing IgG antibody reactive with all normal platelets tested and with autologous platelets, but not with platelets from a patient with BSS. He recovered after two whole blood exchange transfusions and one exchange plasmapheresis and is now well with a normal platelet count. Further details of this case have been published elsewhere.[8]

Purified GPIb GPIb was isolated from Triton-solubilized platelet membranes by affinity chromatography as described by Okita et al.[19]

RESULTS

Platelet aggregation. As can be seen in Fig 1, curve A, addition of AP1 to PRP and autologous plasma inhibited ristocetin-induced agglutination. PRP plus autologous PPP or anti-Pl^{A1} antiserum agglutinate normally (curves B and C). PRP plus DIL plasma obtained during illness (D) or postrecovery (E) did not inhibit ristocetin-induced agglutination. DIL also failed to spontaneously aggregate platelets (curve F) or inhibit ADP-induced (curves I and J) or epinephrine-induced aggregation (not shown). AP2 added to PRP and autologous PPP inhibited both ADP (curve G) and epinephrine-induced (not shown) aggregation. As a positive control, curve H shows ADP-induced aggregation of PRP plus autologous PPP. Interestingly, DIL potentiated agonist-induced agglutination and aggregation (curves D, E, I, and J). No such effect was seen with anti-Pl^{1A} antiserum in the case of ristocetin-induced agglutination (curve B) or with anti-Pl^{E1} and anti-Bak^{a} antisera in the case of ADP-induced aggregation (not shown). The postrecovery sample from DIL, which did not contain platelet-binding antibody as determined by PSIFT, ^{51}Cr release, and electroimmunoassay, also potentiated agonist-induced agglutination and aggregation (curves E and J). This argues against the possibility that this phenomenon is antiplatelet antibody mediated.

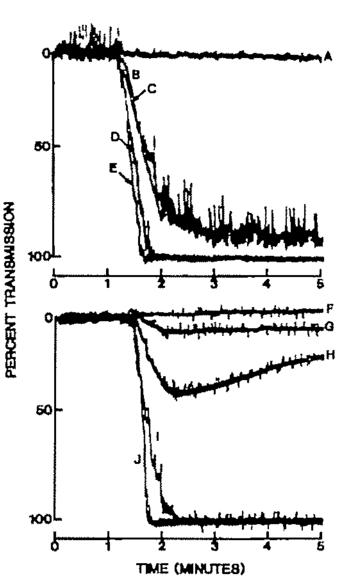

Fig 1. **Ristocetin-induced agglutination of platelets incubated with various antisera is shown in the upper panel. Monoclonal antibody AP1 specific for GPIb inhibits agglutination (A). No inhibition is seen with autologous PPP (B) or with anti-Pl^{A1} (C). Agglutination was enhanced with DIL plasma obtained acutely (D) and postrecovery (E). ADP-induced aggregation of platelets incubated with various antisera is shown in the lower panel. DIL plasma (acute phase) did not cause spontaneous aggregation of platelets (F). ADP-induced aggregation was inhibited by the monoclonal antibody AP2 (G), but not by autologous plasma (H). ADP-induced aggregation was accentuated by DIL plasma obtained acutely (I) and postrecovery (J).**

Immunofluorescence testing. Autoantibody DIL reacted strongly with platelets from each of 50 normal subjects in the PSIFT using a fluorosceinated polyspecific antiimmunoglobulin reagent. Equally strong reactions were obtained using autologous platelets obtained after recovery. DIL reacted strongly against platelets from a patient with type I Glanzmann's thrombasthenia (GT), but failed to react with platelets from a patient with BSS (Table 1).

The patient with GT was described by Kunicki and Aster[20] and designated type I according to the criteria of Caen.[21] This patient had normal platelet levels, prolonged bleeding time and an absence of clot retraction and lacked ADP-inducible platelet aggregation. It was also demonstrated in our laboratories that the patient's platelets contained less than 1% of normal levels of GPIIb-IIIa (unpublished observations, February 1983).

Table 1. Immunofluorescence Testing of Representative Platelets

	DIL Platelets	Control Platelets	GT Platelets	BSS Platelets
DIL 10/19/80	4+	4+	3+/4+	+/−
DIL 1/19/84 (postrecovery)	+	0	ND	ND
Anti-Pl^{A1}	4+	4+	0	3+/4+
Drug antibody + Quinidine	4+	4+	4+	0
Control serum	+	0	0	+

Abbreviation: ND, not determined.

Representative data using DIL antiserum during thrombocytopenia and postrecovery, anti-Pl^{A1} antiserum, drug antibody plus quinidine, and control serum tested with autologous isologous thrombasthenic and BSS platelets. Results are graded on a 0 to 4+ scale.

The BSS patient met the diagnostic criteria of moderate thrombocytopenia, prolonged bleeding time, reduced platelet-subendothelial adherence and giant platelets in the peripheral blood smear. This patient's diagnosis has been documented in previous studies.[22]

Inhibition of ^{51}Cr release by platelets and platelet fractions. Because DIL has complement-fixing properties, it was possible to test different preparations for the presence of target antigen by determining their ability to interfere with complement-dependent, antibody-mediated release of ^{51}Cr from labeled target platelets. DIL, but not an anti-Pl^{A1} antibody, was partially inhibited by 0.4 μg of purified GPIb in this system (Fig 2). Platelets from a patient with BSS failed to inhibit release induced by DIL or by a quinidine-dependent antiplatelet antibody, but totally blocked the action of anti-Pl^{A1} (Fig 3). Normal platelets (Fig 4) and platelets obtained from patient DIL after recovery readily inhibited all three antibodies (data not shown). Since BSS platelets are known to lack GPIb,[23] these findings provide additional evidence that DIL reacts with GPIb.

Absorption of platelet lysates with antibody-coated SPA. A quantitative electroimmunoassay of platelet lysates[16] absorbed with SPA-Sepharose beads coated with normal IgG, DIL, and anti-Pl^{A1} antibody is shown in Fig 5.

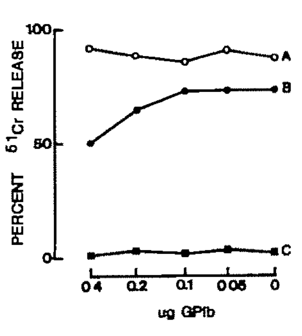

Fig 2. Inhibition of lytic activity of autoantibody DIL by GPIb. Lytic activity of autoantibody DIL (B), as measured by ^{51}Cr release from target platelets, was inhibited by higher concentrations of purified GPIb. Values on the abscissa represent the total amount of GPIb necessary to inhibit the lytic activity in 50 μL of antiserum. Lytic activity of anti-Pl^{A1} (A) was unaffected. C represents a control serum. Results are the mean determination of three experiments.

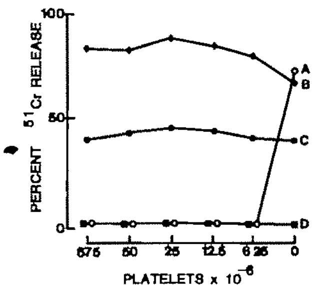

Fig 3. Inhibition of the lytic activity of selected antibodies by BSS platelets. Complete inhibition of anti-Pl^{A1} (A) was achieved at a low concentration of platelets, but even the highest concentration was without effect on autoantibody DIL (C) or a quinidine-dependent antiplatelet antibody (B). D represents a control serum. Values on the abscissa represent the total number of platelets necessary for inhibition of lytic activity of 50 μL of antiserum. Results represent the mean of three experiments.

The platelets were solubilized in the presence of leupeptin to prevent calpain cleavage of GPIb. In side B (lane 2), it can be seen that beads coated with DIL completely removed GPIb as shown by the absence of detectible binding of ^{125}I-labeled monoclonal antibody specific for this glycoprotein (AP1). Beads coated with normal IgG (lane 1) or anti-Pl^{A1} antibody (lane 3) failed to affect GPIb.

Analyses by crossed immunoelectrophoresis are shown in Fig 6. These platelet lysates were prepared in the absence of leupeptin to allow calpain cleavage of glycocalicin from GPIb. From lysates containing GPIb and glycocalicin, beads that were coated with DIL removed GPIb but not glycocalicin (Fig 6B). Beads coated with normal IgG (Fig 6A) or anti-Pl^{A1} (not shown) failed to remove either GPIb or glycocalicin from the lysate. In similar experiments, beads coated with DIL or with normal IgG failed to remove GPIIb-IIIa, whereas beads coated with anti-Pl^{A1} completely removed the GPIIb-IIIa complex (data not shown). This is consistent with our earlier finding that the Pl^{A1} antigen is expressed on GPIIIa.[24]

DISCUSSION

The serum used in our studies came from a patient with an unusual form of ITP characterized by a potent complement-

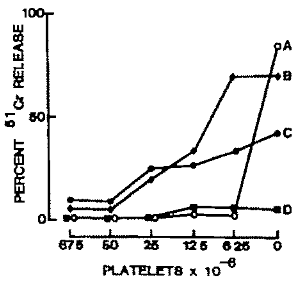

Fig 4. Inhibition of the lytic activity of selected antibodies by type O control platelets. Lytic activity of anti-Pl^{A1} (A), DIL (C), and a quinidine-dependent antiplatelet antibody (B) is inhibited by normal control platelets. D represents a control serum. Platelets obtained from patient DIL after recovery also inhibited all three antibodies (data not shown). Values represent the total number of platelets necessary for inhibition of antibody activity.

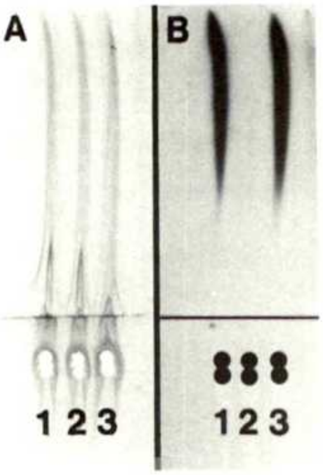

Fig 5. (A and B) One-dimensional electroimmunoassay of Triton-solubilized platelets absorbed with staphylococcal protein A–Sepharose beads coated with normal IgG (lane 1), autoantibody DIL (lane 2), and anti-Pl^{A1} antibody (lane 3). Side A represents Coomassie blue–stained gel containing radiolabeled monoclonal AP1 specific for glycoprotein Ib; side B is the autoradiogram of A. The polyclonal antibody used to generate immunoprecipitates was produced in rabbits. Removal of GPIb by autoantibody DIL is evident from inspection of the autoradiogram (lane 2). Removal of the prominent peak corresponding to GPIIb-IIIa by anti-Pl^{A1} can be seen in the Coomassie blue–stained preparation on the left (lane 3).

fixing antibody that reacted equally well with autologous and isologous platelets. Immunofluorescent studies showed that the patient's serum contained platelet-specific antibodies of the IgG class that reacted with platelets from 50 randomly selected normal donors regardless of HLA or platelet phenotype and with autologous platelets, but failed to react with BSS platelets,[8] which lack GPIb.[23]

Because BSS platelets lack the receptor necessary for interaction with ristocetin/human von Willebrand factor (vWf) and therefore with subendothelium,[22] it has been suggested that GPIb is that receptor. It has also been shown that Ib binds thrombin[25] and drug-dependent antiplatelet antibodies.[26] GPIb is a major platelet membrane glycoprotein composed of a disulfide-linked 143,000-dalton alpha chain and a 23,000-dalton beta chain.[27] When GPIb is exposed to calpain, a 135,000-dalton derivative of the alpha chain, glycocalicin, is released.[28,29]

Because an alloantibody directed against GPIb from a multiply transfused patient with BSS[30] and monoclonal antibodies against glycocalicin are able to inhibit ristocetin/human vWf agglutination[31,32] and adhesion to subendothelium,[30] the possibility exists that an autoantibody against Ib could also cause functional defects. Since patient DIL was profoundly thrombocytopenic, it is likely that his bleeding defect was due to a reduced number of platelets rather than to a functional abnormality. In support of this, autoantibody DIL did not inhibit or promote ristocetin-induced platelet agglutination (Fig 1). The monoclonal antibodies capable of inhibiting ristocetin agglutination recognize glycocalicin, whereas DIL does not. This finding is consistent with the view that the glycocalicin portion of GPIb is responsible for this function. Although autoantibody DIL did promote ristocetin- and ADP-induced agglutination and aggregation, it is unlikely that these phenomena played a role in his disease since the postrecovery sample produced the same effect.

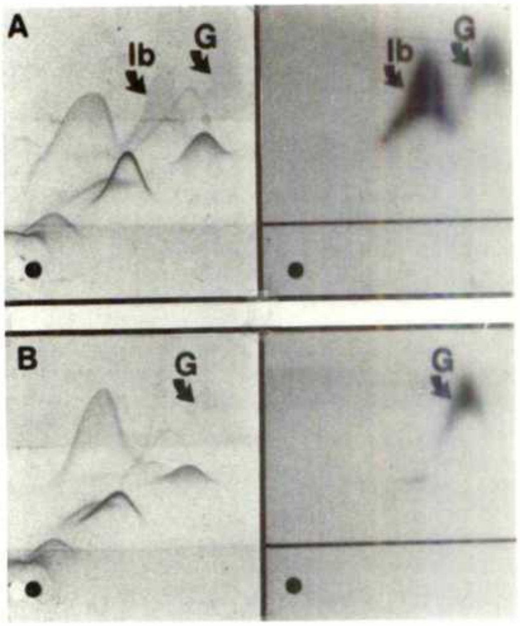

Fig 6. Crossed immunoelectrophoresis of platelet proteins solubilized in the absence of leupeptin and absorbed with SPA-Sepharose beads coated with normal IgG (A) and auto-antibody DIL (B). The precipitating antibody was rabbit antihuman platelet membrane. Gels also contained radiolabeled monoclonal antibody AP1 specific for GPIb; the left half of each panel shows a Coomassie blue–stained preparation, and the right half shows the corresponding autoradiogram. (A) shows GPIb and glycocalicin that was cleaved and released from GPIb by calpain. Removal of the peak corresponding to GPIb, but not glycocalicin, by autoantibody DIL is apparent in (B).

The aforementioned reports suggest that autoantibodies of this type are relatively rare in ITP. In this connection, several features of the case of Mr DIL are of interest. (1) Mr DIL presented with life-threatening thrombocytopenia and responded dramatically to whole blood and plasma exchange transfusion after other therapeutic measures failed. (2) Autoantibody DIL was capable of activating complement in vitro upon binding to platelets as demonstrated in the ^{51}Cr release test. We are aware of only one other case in which autoantibody in the serum of a reported patient with ITP was clearly shown to have this property.[33] (3) Mr DIL had been taking quinidine for about 2 months before the onset of thrombocytopenia, yet extensive studies failed to demonstrate any dependence upon quinidine for binding of antibody DIL to platelets.[8] It may be significant that many, if not all, quinidine- and quinine-dependent platelet-reactive antibodies bind to GPIb at a site proximal to the site of cleavage by papain or chymotrypsin.[28] In some patients, it is possible that quinidine induces true autoantibodies that bind to a site on the GPIb molecule close to that recognized by the more commonly encountered drug-dependent antibodies.

Specificity of this antibody for a site on GPIb was suggested by its ability to precipitate a 160,000 to 170,000-

Dalton protein from radioiodinated platelet lysates (data not shown), by its failure to react with BSS platelets (Table 1 and Fig 3), and by partial inhibition of its cytotoxic activity by purified GPIb (Fig 2). Direct evidence that this autoantibody recognizes GPIb was provided by the demonstration that, when bound to SPA, it can remove all material in detergent-solubilized platelets reactive with AP1 as demonstrated in one-dimensional gels (Fig 5). In crossed immunoelectrophoresis studies with lysates prepared in the absence of leupeptin, it was found that DIL removed the high–molecular weight form of GPIb from this preparation but not the glycocalicin moiety cleaved from GPIb by the action of calpain (Fig 6). The specificity of these reactions was established by showing that DIL has no effect on GPIIb-IIIa, that anti-Pl^{A1} antibody completely removes GPIIb-IIIa but not GPIb and that normal IgG fails to affect either group of proteins in the same experimental systems.

These findings provide direct evidence that autoantibody DIL recognizes a determinant on GPIb located on the membrane side of the site at which calpain acts to cleave glycocalicin from this glycoprotein.

ACKNOWLEDGMENT

We wish to thank Dr Robert R. Montgomery, for his scientific advice and counsel throughout the course of these studies and Margaret M. Johnson for kindly providing the BSS cells. We would also like to thank Susan Kristopeit for technical assistance and Elizabeth Vokac for maintenance of the hybridoma laboratory.

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Expression of Interleukin-2 Receptor on T Cell Chronic Lymphocytic Leukemia Cells and Their Response to Interleukin-2

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We analyzed peripheral blood leukemic cells from six patients with T cell chronic lymphocytic leukemia (T-CLL) with monoclonal antibodies including the anti-Tac antibody, which recognizes the receptor for interleukin 2 (IL 2). The patients were divided into two groups according to the reactivity of the monoclonal antibodies. Leukemic cells from three patients with T-CLL reacted with OKT3 and T4 but not T8, whereas those from the remaining three patients reacted wtih OKT3 and T8 but not T4. IL 2 receptor, which is expressed on activated T cells but not on resting T cells, was preferentially expressed on T4+ T-CLL cells. The IL 2 receptor on T4+ T-CLL cells was indistinguishable from that on normal activated T cells with respect to molecular weight and downregulation by the anti-Tac antibody. Moreover, fresh T4+ T-CLL cells, but not T8+ T-CLL cells, proliferated in response to exogenous IL 2 without prior activation, and this proliferation was inhibited by the anti-Tac antibody. These results suggest that malignant growth of T4+ T-CLL cells can be regulated by IL 2 not only in vitro but also in vivo.

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RECENT PROGRESS in cellular immunology revealed that peripheral blood T lymphocytes are a mixed population consisting of different functional subsets that are defined by the monoclonal antibodies to T cell surface antigens.[1-3] Also, it was found that the interaction of lymphocytes is mediated by some lymphokines, in particular, T cell proliferation is mediated by a 15,000-dalton polypeptide hormone designated interleukin 2 (IL 2).[4,5] As described elsewhere,[6] the prior expression of specific receptors for IL 2 on the T cell membrane is required for IL 2–induced proliferation. The IL 2 receptor is expressed on the T cells activated with lectins or antigens, probably through triggering of the T cell receptor.[7]

These advances enable us to analyze more precisely the leukemic cells in T cell chronic lymphocytic leukemia (T-CLL) and have given us deeper insights into the pathophysiology of this disorder. T-CLL, which has been considered to be a monoclonal expansion of mature peripheral T cells, is a heterogeneous disease clinically, morphologically, and immunologically.[8-12] In the present studies, we examined the phenotype of leukemic T cells from six patients with T-CLL using monoclonal antibodies including the anti-Tac antibody, which recognizes the IL 2 receptor and competitively inhibits IL 2–induced T cell proliferation.[13-15] In addition, the proliferative response of fresh T-CLL cells to exogenous IL 2 was studied. We show here that leukemic cells with an OKT3+, -4+, and -8− phenotype express the IL 2 receptor and proliferate in response to IL 2, whereas leukemic T cells with an OKT3+, -4−, and -8+ phenotype neither express the IL 2 receptor nor respond to IL 2. We will discuss the implications of the IL 2 receptor expression in T-CLL cells compared with that in adult T cell leukemia (ATL) cells,[16,17] which constitutively express the IL 2 receptor.[18,19]

MATERIALS AND METHODS

Patient population. Six patients with T-CLL and seven normal individuals were studied. The age of T-CLL patients ranged from 39 to 69 years, and all patients were male. The diagnosis of T-CLL was made collectively considering clinical features, hematologic characteristics, and cell surface phenotypes.[8-12] ATL endemic in southwest Japan was ruled out on the basis of its unique features[16] and serum antibodies to ATL-associated antigens (ATLA).[20]

Monoclonal antibodies. The OKT3, -T4, -T6, -T8, -T9, -T10, -T11, and Ial antibodies were obtained from Ortho Pharmaceutical Corp (Raritan, NJ); their production and reactivities are described elsewhere.[1-3] The IL 2 receptor on the cell surface was detected by the anti-Tac monoclonal antibody. The IgG fraction of the anti-Tac antibody from hybridoma ascites was purified by gel and diethyl aminoethyl (DEAE) cellulose chromatography.

Analysis of cell surface markers. Peripheral blood mononuclear cells (PBMC) were separated from heparinized peripheral blood by Ficoll-Hypaque density gradient centrifugation. Cell surface antigens were detected by the indirect immunofluorescence method and flow cytofluorometry. Cells (1×10^6) were incubated with saturating amounts of monoclonal antibodies for 30 minutes at 4 °C. After washing twice with Hanks' balanced salt solution containing 0.1% sodium azide and 0.1% bovine serum albumin (BSA), the cells were stained with saturating amounts of a fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ fraction of goat antimouse IgG (Cappel Laboratories, Cochranville, Pa) for 30 minutes at 4 °C, then washed twice. Analysis was performed using Spectrum III (Ortho Diagnostics, Westwood, Mass).

To determine whether Tac+ T-CLL cells are included in the T4+ population, a two-color fluorescence analysis was performed. Cells were sequentially stained (with thorough washing after each reagent) with 5 μL of FITC-conjugated OKT4, 10 μg/mL of biotinylated anti-Tac antibody, and 5 μL of phycoerythrin-conjugated avidin (Avidin PE, Becton Dickinson, Mountain View, Calif) and passed on Spectrum III. The data were analyzed by an Ortho Model 2140 computer and displayed as dot plots.

Proliferative response of T-CLL cells to IL 2. IL 2, purified from culture supernatants of the Jurkat cell line by an affinity column coupled with the anti–IL 2 monoclonal antibody, was generously provided by Dr K.A. Smith (Dartmouth Medical School, Hanover, NH).[21] The quantity 1 U/mL of IL 2 represents 500 pmol/L or 7.5 ng/mL. In some experiments we used recombinant IL 2 (provided by Takeda Chemical Industries, Osaka, Japan). Both IL 2 showed a similar proliferative effect in the same conditions. T-CLL

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cells (1×10^5) in 200 μL of RPMI 1640 medium containing 10% fetal calf serum (FCS) (M.A. Bioproducts, Walkersville, Md) and 30 μg/mL of gentamicin were cultured in the presence of a twofold dilution series of IL 2 in triplicate in a 96-well plate for 72 hours at 37 °C under 5% CO_2. Proliferation was measured by the incorporation of 0.5 μCi/well of ^{3}H-thymidine (2 Ci/mol, Amersham Japan, Tokyo) during the last six hours. For the controls of a positive and a negative response to IL 2, we used activated normal PBMC cultured with 0.1% phytohemagglutinin (PHA-P, Difco Laboratory, Detroit) for seven days and fresh PBMC, respectively. PHA blasts from five normal individuals who expressed the IL 2 receptor[6] proliferated remarkably in response to IL 2. In contrast, fresh PBMC from three normal individuals with no detectable IL 2 receptor showed a minimum proliferative response. In experiments of the inhibition of IL 2–induced proliferation by the anti-Tac antibody, the antibody (100 to 10^{-2} μg/mL) was added to the triplicate cultures of T-CLL cells (1×10^5/well) plus 0.25 U/mL of IL 2. The percent inhibition was calculated by the following formula: percent inhibition = 100 × [1 − (cpm experimental − cpm medium control)/(cpm IL 2 control − cpm medium control)], where the medium control is the culture with medium alone (271 ± 29 cpm for patient 1 and 328 ± 69 for patient 2) and the IL 2 control is 12,747 ± 974 cpm for patient 1 and 18,839 ± 1,935 for patient 2.

Downregulation of IL 2 receptor by anti-Tac antibody. We examined the effect of anti-Tac antibody on the expression of the IL 2 receptor on T-CLL cells from patient 1 by the methods described previously.[18,19] Briefly, cells (1×10^6/mL) were cultured for 48 hours in the presence or absence of 10 μg/mL of anti-Tac antibody. Then, the resulting expression of IL 2 receptor was detected by the indirect immunofluorescence method described previously.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The Tac antigen on normal PHA blasts and T-CLL cells from patient 1 was analyzed by SDS-PAGE according to the methods described previously.[22] Briefly, cells were surface labeled with Na(^{125}I) (New England Nuclear, Boston) by the lactoperoxidase method and lysed in an extraction buffer (0.5% NP-40, 0.15 mol/L NaCl, 10 mmol/L Tris-HCl, pH 7.2). Tac antigen was immunoprecipitated with anti-Tac antibody and protein A–Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ). The immunoprecipitate was electrophoresed on 7.5% polyacrylamide gel and visualized by autoradiography.

RESULTS

Clinical and hematologic features of T-CLL patients. Table 1 shows the clinical data of six patients with T-CLL. Peripheral lymph node enlargement was observed in four of six patients, whereas no mediastinal mass was noted roentgenologically. Patients 5 and 6 presented with hepatosplenomegaly. Skin involvement confirmed by biopsy was seen in three patients; patient 2, in addition, had generalized erythroderma similar to that seen in the Sézary syndrome. However, we made a diagnosis of T-CLL since the leukemic cells were morphologically different from Sézary cells and expressed the Tac antigen, which is negative in the Sézary syndrome,[23] as will be described. Patients 2, 5, and 6 died from gastrointestinal bleeding, renal failure because of infiltration of leukemic cells, and pulmonary infection, respectively. All cases showed prolonged lymphocytosis, and WBC counts ranged from 13,500 to 291,000/μL with 70% to 91% lymphoid cells. Mild anemia was seen in patients 3 and 6 at the time of diagnosis. Bone marrow infiltration of leukemic cells was relatively mild except in patient 6. Typical morphologic features on May-Giemsa stain are shown in Fig 1. Leukemic cells from patients 1, 5, and 6 were slightly larger than RBC and had coarsely clumped nuclear chromatin with scant cytoplasm. In patient 5 especially, leukemic cells had lobulated nuclei similar to that seen in ATL cells. Leukemic cells from patients 2, 3, and 4 were large in size and had abundant cytoplasm with azurophilic granules in variable amounts. To rule out ATL, the sera of patients with T-CLL in the present study were analyzed for antibodies to ATLA according to the method described previously.[20] None of the patients had anti-ATLA antibodies.

Table 1. Clinical Features of T-CLL

Patient	Age	Sex	Survival Time (months)	Organomegaly			Skin Lesion	Bone Marrow Lymphoid Cells (%)
				Lymph Node	Liver	Spleen		
1 O.O.	54	m	>30	+	−	−	+	28
2 S.K.	62	m	9	+ +	−	−	+ +	40
3 D.T.	69	m	>28	−	−	−	−	30
4 F.Z.	39	m	>33	−	−	−	−	18
5 H.K.	65	m	19	+	+	+	+	23
6 H.S.	57	m	30	+	+	+ +	−	76

Surface markers. Cell surface markers of the patients are summarized in Table 2. Leukemic cells from all cases displayed the T3 antigen, suggesting that these leukemic cells arise from peripheral mature T cell subsets. OKT6 and -T9 antibodies, which define thymocytes and the transferrin receptor, respectively, were unreactive in all cases. Of interest was the finding that the patients clearly fell into two groups depending on their reactivity with OKT4 and -T8 antibodies, which define the helper/inducer and suppressor/cytotoxic T cell subsets, respectively. Major populations of T-CLL cells from patients 1 to 3 were T4+, T8−. In contrast, T-CLL cells from patients 4 to 6 were T4−, T8+.

Expression of IL 2 receptor on T-CLL cells. We then investigated these leukemic cells using an anti-Tac monoclonal antibody that recognizes the IL 2 receptor. It is noteworthy that a proportion (23%, 4%, and 7% in patients 1 to 3, respectively) of fresh peripheral blood lymphoid cells from T4+ T-CLL patients expressed the Tac antigen, whereas those from T8+ T-CLL patients were obviously negative for the Tac antigen (Table 2).

Next, reactivity with anti-Tac was studied after cultivation (10^6 cells/mL) with 2 U/mL recombinant IL 2 or medium alone for two days and compared with normal PBMC. As shown in Table 3, although the number of Tac+ cells for patients 2 and 3 was low, it increased remarkably in all T4+ T-CLL cases, but not in T8+ T-CLL. However, normal PBMC expressed the Tac antigen on only 9% of cells by stimulation of IL 2, suggesting that the increase of Tac+ cells in T4+ T-CLL was attributed to leukemic cells, but not to normal contaminated cells. In addition, the two-color fluorescence analysis depicted in Fig 2 clearly shows that almost all Tac+ cells were included in the T4+ population. Taken together, these results indicate that Tac+ cells represent T4+ leukemic cells.

Proliferative response to IL 2. To elucidate whether the Tac antigen on T-CLL cells is a functional IL 2 receptor, we

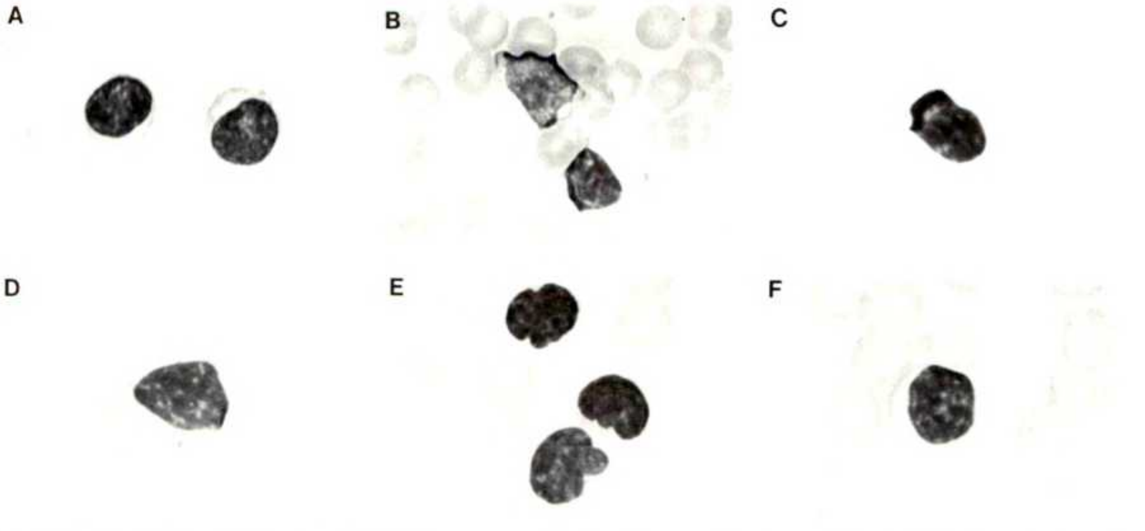

Fig 1. Leukemic cells in peripheral blood using May-Giemsa stain (original magnification ×1000). Numbers correspond to patients' numbers. (A), Patient no 1; (B), Patient no. 2; (C), Patient no. 3; (D) Patient no. 4; (E), patient no. 5; (F), Patient no. 6.

studied the proliferative response of T-CLL cells to affinity-purified IL 2 as described in Materials and Methods (Fig 3). Leukemic cells from the T4+ T-CLL patients showed a good proliferative response to IL 2, comparable to that of normal PHA blasts used as a positive control. In contrast, leukemic cells from the patients with T8+ T-CLL, which did not express the Tac antigen, showed no significant proliferation when cultured with IL 2 for three days. To assess whether the proliferation of T4+ T-CLL cells by IL 2 was induced via the Tac antigen/IL 2 receptor, leukemic cells from patients 1 and 2 were cultured with 0.25 U/mL of IL 2 in the presence of the anti-Tac antibody or control mouse myeloma (HOPC-1) IgG at various concentrations. As shown in Fig 4, IL 2–induced proliferation was remarkably inhibited by the anti-Tac antibody.

Downregulation of the IL 2 receptor by the anti-Tac antibody. Our recent study[19] disclosed that the expression of the IL 2 receptor is regulated by an external ligand. As is the case of the common acute lymphocytic leukemia antigen (CALLA)[24] and the acetylcholine receptor,[25] the IL 2 receptor on normal T cell blasts is modulated or downregulated by the anti-Tac antibody. However, the regulation mechanism of the IL 2 receptor by the anti-Tac antibody is lacking in ATL in which the IL 2 receptor is continuously expressed without exogenous stimuli. Thus, we investigated whether the expression of the IL 2 receptor on T4+ T-CLL cells is affected by the anti-Tac antibody. Unlike ATL, the IL 2 receptor on T4+ T-CLL cells from patient 1 was clearly downregulated when cultured with 10 μg/mL of anti-Tac antibody (Fig 5), suggesting that the regulation mechanism of the IL 2 receptor as well as the response to IL 2 is preserved in T4+ T-CLL.

Table 2. Cell Surface Markers of T-CLL

Patient	WBC (× 10⁻³/μL)	Lymphoid Cells (%)	Positive Cells (%)									
			OKT3	T4	T6	T8	T9	T10	T11	Ia1	sIg	Tac
1	30.1	70	87	84	0	3	1	—*	86	8	2	23
2	291.0	90	91	76	0	5	4	—	—	84	5	4
3	15.9	74	93	91	—	10	1	8	26	42	8	7
4	13.5	91	90	2	—	98	0	1	—	0	2	0
5	32.0	88	97	1	0	98	0	—	94	29	1	0
6	22.8	90	92	9	0	74	1	33	95	30	—	0

*Not tested.

SDS-PAGE analysis. The IL 2 receptor expressed on T4+ T-CLL cells from patient 1 was analyzed by surface radioiodination, immunoprecipitation with anti-Tac, and then PAGE. As shown in Fig 6, the IL 2 receptor on T4+ T-CLL cells exhibited the same band (60 to 65 kilodaltons) as that of PHA-activated normal T cells.

DISCUSSION

In this report, we analyzed six cases of T-CLL by their reactivity with monoclonal antibodies including the anti-Tac antibody and the proliferative response of leukemic cells to IL 2. Many investigators have noted that T-CLL is a lymphoproliferative disease of mature T cell origin and a heterogeneous disease clinically, morphologically, and immunologically.[8-12] When examining patients in Japan with prolonged lymphocytosis of mature-appearing T cells it is necessary to consider ATL, which is endemic in southwest

Table 3. Expression of Tac Antigen on T-CLL Cells

Patient	Tac+ Cells (%)		
	Fresh	Cultured With	
		Medium Alone	IL 2*
1	23	59	80
2	4	7	34
3	7	6	30
4	0	0	0
5	0	ND	ND
6	0	0	2
Normal PBMC (n = 7, mean ± SEM)	0 ± 0	4 ± 2	9 ± 3

Abbreviation: ND, not determined.

*Cells were cultured with 2 U/mL of recombinant IL 2 for two days.

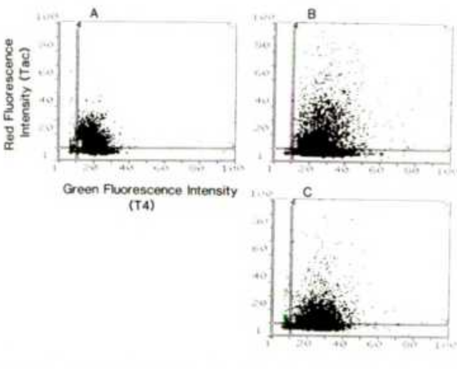

Fig 2. Two-color fluorescence analysis of T4+ T-CLL cells. After culture with recombinant IL 2 for two days, T-CLL cells were stained with OKT4 (green fluorescence) and anti-Tac (red). Tac+ cells were included in the T4+ population. Sections A, B, and C correspond to patients 1, 2, and 3, respectively.

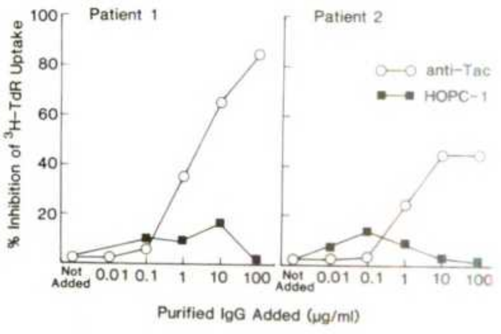

Fig 4. The inhibition of IL 2-induced proliferation of T-CLL cells by the anti-Tac antibody. Leukemic cells (1×10^5/well) from patients 1 and 2 were cultured with 0.25 U/mL of IL 2 in the presence of anti-Tac (○) or control IgG (■) from HOPC-1 mouse myeloma cells. Data represent the percent inhibition of ^{3}H-thymidine incorporation.

Japan and has a phenotype of mature helper/inducer T cells (OKT3+, -T4+, and -T8−).[16,17] Virtually all ATL patients have serum antibodies to ATLA[20] and provirus integration of the human T cell leukemia virus (HTLV-I) in the DNA of leukemic cells.[26,27] Our six cases, however, did not have anti-ATLA antibodies. Moreover, patients 1, 2, 5, and 6 tested showed no proviral integration when examined by Southern blot hybridization (Dr M. Maeda, Kyoto University, personal communication, Jan 10, 1985).

The results of our phenotypic study of six T-CLL patients shows that all had a phenotype of mature T cells: they were positive for the T3 antigen, specific to mature peripheral T cells, but negative for the T6 and T9 (transferrin receptor) expressed on thymocytes and rapidly dividing cells, respectively. It was of interest that three patients had a phenotype of helper/inducer T cells (T4+, T8−), whereas the other three patients had a phenotype of suppressor/cytotoxic T cells (T4−, T8+). These results are consistent with previous reports from other laboratories.[11,12]

Accumulating evidence indicates that proliferation of T cells is regulated by IL 2 and its receptors.[4-6] The IL 2 receptor, recognized by the anti-Tac antibody, is expressed transiently on T cells activated with lectins and antigens but not on resting T cells. Our observations showed that T4+ T-CLL expressed the IL 2 receptor with variable extent and that the IL 2 receptor was induced or upregulated when cultured with IL 2 for two days. The upregulation of the IL 2 receptor has been reported in the system of concanavalin A-stimulated normal blasts by Reem and Yeh.[28] In patient 1, it was clear that both fresh and IL 2-stimulated leukemic cells expressed the IL 2 receptor. In patients 2 and 3, however, it was not clear that Tac+ cells in fresh peripheral blood lymphoid cells really represent leukemic cells because the percentage of Tac+ cells was low (4% and 7%). In one of the experiments to elucidate this issue, we examined Tac+ cells after culture with IL 2, comparing T4+ T-CLL cells with normal PBMC. The results made it most unlikely that Tac+ cells in patients 2 and 3 were contaminated normal T cells or natural killer (NK) cells because the two-color fluorescence analysis revealed that virtually all Tac+ cells were included in the T4+ population. And normal lymphocytes, if contaminated in T4+ T-CLL cells, would not express the Tac antigen by culture with IL 2 for two days since very little Tac antigen was expressed on normal PBMC cultured under the same conditions. Collectively, we concluded that leukemic cells expressed the IL 2 receptor.

In contrast to T4+ T-CLL, neither fresh nor IL 2-stimulated cells expressed the IL 2 receptor in T8+ T-CLL patients. It is unlikely that normal T8+ cells essentially have

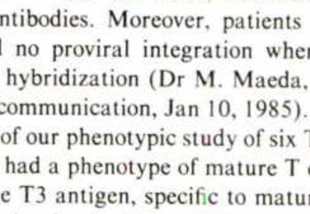

Fig 3. Proliferative response of T-CLL cells to IL 2. Leukemic cells (1×10^5/well) of T4+ T-CLL (patients 1, 2, and 3) proliferated in response to IL 2, whereas those of T8+ T-CLL patients (patients 4, 5, and 6) showed no significant proliferation. Zones A and B represent the mean ± SEM of the IL 2 response of PHA blasts (n = 5) and fresh PBMC (n = 3) from normal individuals, respectively.

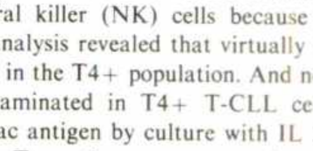

Fig 5. Downregulation of the IL 2 receptor on T-CLL cells by the anti-Tac antibody. The fluorescence profile of the IL 2 receptor on T-CLL cells from patient 1 is charted: (A) fresh leukemic cells, (B and C) leukemic cells cultured with medium alone or 10 µg/mL of anti-Tac antibody for 48 hours, respectively.

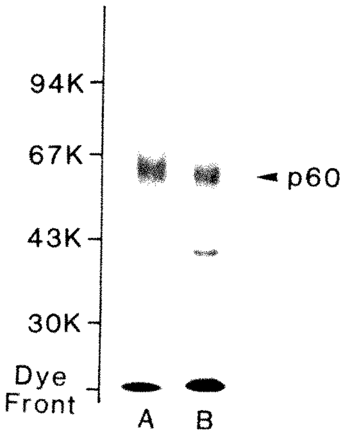

Fig 6. SDS-PAGE analysis of the IL 2 receptor on normal PHA blasts (A) and T-CLL cells (B) from patient 1. Cells were surface iodinated, solubilized, and then immunoprecipitated by anti-Tac. Migration of molecular weight markers is shown at the left. The 42 K sharp band on lane B may be actin.

no capacity to express the IL 2 receptor because some IL 2–dependent T8+ lines have been reported.[29,30] Thus, the expression of the IL 2 receptor on leukemic cells appears to be one of the characteristics of T4+ T-CLL.

It is important, then, to determine whether IL 2 can regulate the malignant growth interacting with the IL 2 receptor on leukemic cells. Our results clearly show that T4+ T-CLL cells proliferated in response to the exogenous IL 2 dose dependently, whereas T8+ T-CLL cells bearing no IL 2 receptor did not. It is reasonable to speculate that since major cell populations from T4+ T-CLL patients were negative for the IL 2 receptor a good proliferative response comparable to that of normal PHA blasts might not be obtained. However, we observed that the percentage of Tac+ cells in T4+ T-CLL remarkably increased when cultured with IL 2. Collectively, leukemic cells from T4+ T-CLL, unlike T8+ T-CLL, had functional IL 2 receptors and proliferated in response to IL 2, suggesting that malignant proliferation can be regulated by IL 2 not only in vitro but also in vivo. Moreover, these results suggest the use of the anti-Tac antibody that inhibits the IL 2–induced proliferation of leukemic cells for therapy of T4+ T-CLL bearing the IL 2 receptor.

In regard to the IL 2 receptor, it is very interesting to compare T4+ T-CLL with ATL, which has a phenotype of mature/inducer T cells and expresses IL 2 receptors.[17,23] The first point is the downregulation of the IL 2 receptor by the anti-Tac antibody. We reported previously[18] that the IL 2 receptor on normal activated T cells was modulated or downregulated when cultured with anti-Tac antibody for 48 hours. This downregulation, however, could not be observed in the time frame usually used to study ligand-receptor downregulation (minutes to two to three hours) (unpublished data), suggesting that the mechanism may be different from that of CALLA or the mouse TL antigen. We showed in the present study that the IL 2 receptor on T4+ T-CLL cells in patient 1 was downregulated by anti-Tac. In contrast to the pattern of normal T cells and T4+ T-CLL cells, the IL 2 receptor on ATL cells cannot be downregulated by anti-Tac.[19] Our studies[31] using ^{125}I–anti-Tac have revealed that the number of anti-Tac binding sites per cell for normal PHA blasts, patient 1, five ATL patients, and ATL-derived HUT 102 cell line cells were 35,000; 2,000 to 8,000; 3,100 to 11,400; and 460,000, respectively. Neither the Tac antigen (460,000/cell, much more than in PHA blasts) on HUT 102 cells nor that (3,100 to 11,400/cell, less than in PHA blasts) on ATL cells was downregulated. Thus, there seems to be no correlation betwen the downregulation and the number of Tac antigens. In addition, it should be emphasized that ATL cells in most cases showed no proliferative response to added IL 2.[31] Therefore, although the SDS-PAGE analysis of the Tac antigen failed to disclose differences between T-CLL and ATL,[31] we speculate that the IL 2 receptor on T-CLL cells has a property similar to that of normal activated T cells but not to that of ATL cells.

Recently, Gallo and Wong-Staal[32] proposed an autocrine theory for the oncogenesis of ATL. They hypothesized that HTLV-infected T cells are activated to produce IL 2, resulting in the continuous stimulation of their own IL 2 receptor. They later ruled out their hypothesis because of the lack of IL 2 gene expression in HTLV-infected cells.[33] However, although the study of IL 2 production by leukemic cells remains to be done, our results suggest that at least in some T4+ T-CLL cases an autocrine theory may be applicable, in part, to explain the neoplastic growth of T cells.

ACKNOWLEDGMENT

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Platelet Adhesion and Thrombus Formation on Subendothelium in Platelets Deficient in Glycoproteins IIb-IIIa, Ib, and Storage Granules

By Harvey J. Weiss, Vincent T. Turitto, and Hans R. Baumgartner

Patients whose platelets are deficient in glycoprotein (GP) Ib, IIb-IIIa (thrombasthenia), or granule substances (storage pool deficiency, SPD) were studied to define further the properties of platelets that mediate platelet adhesion and thrombus formation on subendothelium. Both nonanticoagulated and citrated blood were exposed to everted, de-endothelialized rabbit vessel segments under controlled flow conditions and shear rates varying from 650 to 3,300 sec^{-1}. Morphometry was used to measure platelet thrombus dimensions and the percentage of the subendothelial surface covered with contact (C) or spread (S) platelets. Adhesion was defined as C + S. The results in SPD demonstrated (1) reduced thrombus dimensions in δ-SPD (pure dense granule deficiency) in proportion to the magnitude of the dense granule defect; (2) an even greater reduction in thrombus dimensions in patients with combined deficiencies of α and dense granules (αδ-SPD); and (3) impaired platelet adhesion at several conditions in αδ-SPD and, in δ-SPD, a hematocrit-dependent impairment of adhesion in citrated blood at 2,600 sec^{-1}. In thrombasthenia, platelets were present as a monolayer on the subendothelial surface in both nonanticoagulated and citrated blood, indicating an absolute requirement for GPIIb-IIIa in promoting platelet-platelet interaction at all shear rates and perfusion times. Two types of abnormalities in platelet–vessel wall interactions were observed. In nonanticoagulated blood, the percentage of platelets in the C phase was consistently increased at all shear rates, but C + S values were normal. These observations indicate that platelets deficient in GPIIb-IIIa do not spread normally on the subendothelial surface exposed to nonanticoagulated blood. With citrated blood, the C + S value in thrombasthenia was reduced at both 800 and 2,600 sec^{-1}, as in von Willebrand's disease, and a similar degree of reduction (about 50%) was observed in normal blood treated with a monoclonal antibody to GPIIb-IIIa. The findings, together with theoretical considerations, are consistent with an hypothesis that GPIIb-IIIa mediates the spreading of platelets on subendothelium following the initial attachment through GPIb and that GPIIb-IIIa may be considered an adhesion site on the platelet membrane. Abnormalities of GPIIb-IIIa may, depending on the conditions of study, result in either increased values of C platelets or decreased values of C + S. The results of the study further suggest that a complex interaction of platelet granule factors and membrane GP mediate platelet adhesion and thrombus formation.

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PLATELET ADHESION, followed by recruitment of other platelets into growing thrombi, plays a major role both in the primary arrest of bleeding and in the formation of arterial thrombi after vascular injury. Adhesion, as with other cells,[1] may be accomplished by an initial attachment (contact phase) followed by subsequent spreading of the platelet on the surface. Abnormalities in either contact (C) or spreading (S), which are probably governed by different mechanisms,[1] could result in impaired platelet adhesion on a vascular surface. To study platelet adhesion, we have used a system in which either citrated or nonanticoagulated blood is exposed to the subendothelium of rabbit aorta under controlled flow conditions and, through studies on patients with von Willebrand's disease,[2,3] demonstrated that platelet adhesion (in particular, the initial attachment of platelets to the vessel wall[4]) is mediated by plasma[2,3] or vessel wall[5] von Willebrand factor (vWF). In addition, through studies on patients with the Bernard-Soulier syndrome[3,6] we have demonstrated a role for platelet membrane glycoprotein Ib (GPIb), the vWF receptor that is deficient in this disorder.[7] These observations have been confirmed[8,9] and extended by demonstrating that platelet adhesion is mediated, in part, by the binding of plasma vWF to the vessel wall[9] and by observations that adhesion in normal blood can be reduced by specific antibodies to either vWF[10] or GPIb.[11]

Although the aforementioned evidence strongly suggests that vWF mediates platelet adhesion by binding to GPIb, recent evidence suggests additional possible mechanisms. Thus, several groups have now reported that, in addition to the well-known ability of ristocetin to promote vWF binding to GPIb,[12–17] thrombin and adenosine diphosphate (ADP)-induced vWF binding[16,18–22] is mediated through GPIIb-IIIa.[16,20,22] This binding is decreased in patients with thrombasthenia who are deficient in GPIIb-IIIa[20] and can be blocked by monoclonal antibodies to GPIIb-IIIa.[16] These observations have led Timmons et al to suggest that the binding of vWF to GPIIb-IIIa, perhaps mediated by ADP released from platelet-dense granules,[23] may also play a role in mediating platelet adhesion. However, recent reports demonstrating that this binding can be inhibited by concentrations of fibrinogen normally found in plasma[22,24,25] has raised some question about its physiologic importance.

To explore a possible role for GPIIb-IIIa as well as platelet-dense granule substances such as ADP in mediating platelet adhesion, we have used our perfusion system to study platelet adhesion at several shear rates and exposure times in platelets that are deficient in GPIIb-IIIa and in patients with storage pool deficiency (SPD) who have well-characterized deficiencies of platelet-dense granules. To evaluate the relative contribution of the GPIb and GPIIb-IIIa systems under

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the same conditions, parallel studies were carried out on patients with severe von Willebrand's disease and the Bernard-Soulier syndrome.

In addition, we have also used blood from patients with various types of SPD to examine a possible relationship of platelet α and dense granule defects, and their magnitude, on platelet-platelet interaction (thrombi).

MATERIALS AND METHODS

Perfusion Chamber–Containing Vessel Segments

The perfusion chamber is an annular device on the core of which is mounted an everted segment of rabbit aorta whose endothelium has been completely removed by a balloon catheter.[2,3,6,26] Chambers of two different sizes, designated original and small,[3] were used in the study. Blood entering the chamber passes through the annular space formed by the subendothelial surface and the outer cylinder wall, thereby permitting platelets to interact with the exposed subendothelial surface. The flow parameters, dimensions, and temperature (37 °C) control for the chamber have been previously described.[3,27] Vessel segments either 20 mm (nonanticoagulated blood studies) or 14 mm (citrated blood studies) in length were stored in 0.2 mol/L tris (hydroxymethyl) aminomethane (Tris) buffer, pH 7.4, at 4 °C for periods of seven to 28 days prior to use.

Perfusion Procedure

Everted vessel segments were exposed at varying shear rates and exposure times to citrated or nonanticoagulated blood. Citrated blood, adjusted for the hematocrit value to a final plasma concentration of 19.7 mmol/L sodium citrate, was circulated through the chamber in a closed system using a peristaltic pump (Holter Extracorporeal Medical Specialties, Inc, King of Prussia, Pa) to maintain the desired blood flow.[2,6,26,27] For studies using nonanticoagulated blood, blood from the anticubital vein was drawn directly through an infusion set (Butterfly, Abbott Hospital Products, N Chicago, Ill) into the chamber and was not recirculated.[28] The flow rate was controlled by the Holter pump and was accurately determined after each run by measuring the actual amount of blood perfused.[3]

The wall shear rate for each flow rate used was determined as previously described.[3,27] For flow rates of 10, 20, 40, and 50 mL/min in the small chamber (used for all the studies with nonanticoagulated blood), the corresponding wall shear rates are 650, 1,300, 2,600, and 3,300 sec^{-1}. For the original chamber (used in some of the citrate blood studies), flow rates of 40 and 160 mL/min correspond to wall shear rates of 200 and 800 sec^{-1}.

Morphologic Evaluation

After perfusion, vessel segments were embedded in an oriented manner, and cross sections 0.8 μm in thickness were stained and evaluated morphormetrically by light microscopy.[2-4,6,28] Platelet interaction with the subendothelium was evaluated by two microscopical techniques that were performed on the same cross-sectional area of vessel segment. Platelet interactions were evaluated at a single axial position approximately equidistant from both ends, that is, in studies on nonanticoagulated blood, at a single axial position located 10 mm from the proximal end of the original segment, and in citrated blood studies, at 7 mm from the proximal end. For determining platelet adhesion, the presence of platelets was evaluated at 10-μm intervals, and platelet-subendothelial interaction was evaluated as either C, platelets are attached but not spread on the surface, and S, platelets are spread on the surface and may, in addition, have superimposed aggregates of platelets (thrombi) extending for varying distances into the lumen. Platelet adhesion is defined as C + S, expressed as a percentage of the total number of evaluations (~1,000) per vessel segment. To evaluate more precisely the dimensions of the platelet thrombi, vessel segments are projected onto the recording plate of a manual picture analysis system (MOP/AMOI, Kontron A.G., Zurich, Switzerland).[28,29] The thrombus volume-per-unit surface area is calculated from the total cross-sectional area of all platelet aggregates in a section normalized by the cross-sectional length of the subendothelial surface.[29] Previous studies have shown that the thrombus volume calculated in this manner shows an excellent correlation with the mass of ^{51}Cr-labeled platelets deposited on the surface.[30] The maximum thrombus height is the average peak-to-base distance for the three tallest thrombi in a section. Further details of the MOP technique have been previously published.[29,31]

Subjects

Thrombasthenia. Six patients with platelet abnormalities characteristic of Glanzmann's thrombasthenia were used in various studies. In all patients, the platelets did not aggregate with 5×10^{-5} mol/L ADP and epinephrine or with collagen (20 μg/mL). Three previously reported[32,33] patients (M.C., L.W., M.My.) and another subject, C.G. (kindly studied by Dr Graham Jamieson, American Red Cross, Bethesda, Md), have decreased amounts of GPIIb and IIIa detected by periodic-acid Schiff (PAS) staining after separation of platelet membrane proteins by polyacrylamide gel electrophoresis.[33] Patients M.Mo. and L.M. were kindly referred for study by Dr Margaret Johnson and have been shown previously to have decreased amounts of GPIIb-IIIa.[34] Other studies on the thrombasthenic patients have shown decreased amounts (C.G., 5%; M.Mo., 15%; and L.M., 10% of normal) of GPIIIa in binding studies using a monoclonal antibody to GPIIIa (kindly performed by Dr Perumai Thiagaragan, Jefferson Medical College, Philadelphia) and markedly reduced or absent amounts of the GPIIb-IIIa complex (M.C., L.W., M.Mo., L.M., and C.G.) detectable by crossed immunoelectrophoresis of Triton (Sigma Chemical Co, St Louis) X-100–solubilized whole platelets against a polyclonal rabbit anti-human platelet antibody (kindly performed by Dr Ira Sussman, Montefiore Medical Center, New York). The mean plasma factor VIII–related antigen (VIIIR:Ag) value (±SE) in these patients, determined as previously described,[35] was 1.30 ± 0.15 U/mL (mean normal value, 0.97; range, 0.55 to 1.61).

Storage pool deficiency. Nine patients with SPD had either selective deficiencies of platelet-dense granules (δ-SPD) and dense granule bound substances or were deficient in both dense and α-granules ($\alpha\delta$-SPD).[36] The patients with δ-SPD included six with the albinism variant (δ-SPD [A], Hermansky-Pudlak syndrome] and three unrelated, nonalbino patients (δ-SPD [NA]). Values of platelet ADP, adenosine triphosphate (ATP), and serotonin for most of these patients have been previously reported[36] and, for more recently studied patients, were obtained by previously described methods.[36] Maximal releasable adenine nucleotides (a reflection of the nucleotides specifically stored in dense granules) were obtained by incubating gel-filtered platelets[36] with 5U/mL thrombin at 37 °C and measuring the released ATP and ADP.[36] Average values for patient groups are shown in Table 1 and demonstrate that the most striking dense granule defect (serotonin and secretable adenine nucleotide values) were found in δ-SPD(A) (serotonin values 3% of normal and undetectable adenine nucleotide release), whereas the abnormalities were of a lesser magnitude in δ-SPD(NA) patients (serotonin values 45% of normal, adenine nucleotide release 32% of normal). Five patients with combined deficiencies of both α and

Table 1. Patients With Storage Pool Deficiency

		Adenine Nucleotides (μmol/10^{11} Platelets)				
		Total		Releasable		
Granule Defect	No.	ATP	ADP	(ATP + ADP)	Serotonin (× 10^3)	α-Granules Substances*
δ-SPD						
(A)	6	3.8 ± 0.2	0.2 ± 0.1	0	9 ± 4	normal
(NA)	3	3.9 ± 0.1	0.6 ± 0.1	1.1 ± 0.3	125 ± 35	normal
$\alpha\delta$-SPD						
Family C	4	3.7 ± 0.2	0.7 ± 0.2	0.9 ± 0.3	150 ± 24	decreased
Patient J.C.	1	4.3	0.5	0.7	45	markedly decreased
Normal subjects		4.4 ± 0.2	2.3 ± 0.1	3.4	278 ± 10	

Abbreviations: A, albinos; NA, non-albinos.

*Data from Weiss et al.[36]

Values are mean ± SE.

dense granules[36] were also studied. Four patients are members of a family (family C) with moderate α-granule deficiency, and one unrelated patient (J.C.) is severely deficient in α-granules and granule-bound substances.[36] The dense-granule deficiencies in patients with $\alpha\delta$-SPD[35] were less severe than in patients with δ-SPD(A) (Table 1). The plasma VIIIR:Ag values for SPD patients were as follows: δ-SPD(A), 0.94 ± 0.13; δ-SPD(NA), 0.91 ± 0.11; $\alpha\delta$-SPD (family C), 0.66 ± 0.04; and $\alpha\delta$-SPD (J.C.), 0.62.

Other patients. To compare the results obtained in thrombasthenia and SPD with known defects of platelet adhesion, we studied two previously reported[3,6] patients (T.H. and A.J.) with the Bernard-Soulier syndrome deficient in GPIb,[33] three patients (C.M., D.R., and W.D.) with severe classical von Willebrand's disease who have markedly decreased plasma levels of the FVIII-complex[35] (VIIIR:Ag, <0.03 U/mL by Laurell electroimmunoassay; VIIIR:RCo, <0.03 U/mL; and VIII:C, <0.01 to 0.05 U/mL, assayed as previously described[35]), and one previously reported patient with a marked congenital deficiency of fibrinogen (plasma fibrinogen level, 3 μg/mL).[28] We also studied a patient with another type of platelet membrane defect (Scott syndrome) characterized by an isolated deficiency of platelet procoagulant activity[37] and impaired binding of Factor Xa.[38]

Normal subjects. Control values were obtained by studying normal hospital personnel, ages 25 to 50.

Blood Values

Platelets were counted on blood collected into EDTA by an electronic counting device (model S, Coulter Electronics, Hialeah, Fla). The average values for platelet counts (per μL) were as follows: thrombasthenic patients, 251,000 (187,000 to 340,000); nonalbino patients with δ-SPD(NA), 296,000 (264,000 to 353,000); albino patients with δ-SPD(A), 292,000 (210,000 to 349,000), family C with $\alpha\delta$-SPD, 197,000 (152,000 to 263,000); and patient, J.C., with $\alpha\delta$-SPD, 295,000. The average count in patients with von Willebrand's disease was 262,000 (160,000 to 345,000); in the two patients with the Bernard-Soulier syndrome, 85,000 and 103,000; in the patient with fibrinogen deficiency 300,000; and in the patient with the Scott syndrome (Xa binding defect), 338,000. For the normal subjects used in the study, the average value was 262,000 (178,000 to 355,000). All normal subjects with platelet counts greater than 355,000 were excluded from the study.

Monoclonal Antibody to GPIIb-IIIa

This antibody (M148, a generous gift from Professor Roger Hardisty) was produced by the hybridoma technique against human medulloblastoma and has been reported to be directed, in platelets, specifically against GPIIb-IIIa.[39] It induces functional defects in platelet aggregation that are entirely similar to those observed in patients with thrombasthenia.[39] A control monoclonal antibody (UJ13A) known not to bind to platelets[39] was also studied.

Statistics

The significance of differences between mean values was evaluated by Student's *t* test, adjusting, where appropriate, for small sample sizes and inhomogeneity of variances,[40] and the correlation coefficient (*r*) between two variables from the least-square regression line.

RESULTS

Studies Using Nonanticoagulated Blood

In these studies, venous blood was drawn immediately through the annular perfusion chamber for varying periods of time, and the wall shear rate was controlled by adjusting the flow rate. The various parameters of platelet–vessel wall interaction were determined as described in Materials and Methods and included C platelets, S platelets, C + S, thrombus volume per subendothelial surface area, and average maximum thrombus height.

Normal subjects. Analysis of the vessel segments after perfusion of normal blood revealed the presence of platelets attached to the subendothelial surface in varying stages of activation, including a small percentage that had contacted the surface but had not spread, a larger percentage that had spread and degranulated, and platelet thrombi on top of S platelets.

Thrombasthenia. In patients with thrombasthenia, platelets were observed on the subendothelium as a monolayer of both C and S platelets. The absence of thrombi is similar to findings previously reported using citrated blood[41] and indicate a complete absence of platelet-platelet interaction in thrombasthenia even when the studies are performed without the use of anticoagulants.

Values obtained for C + S in thrombasthenia are shown in Fig 1 (top) for different shear rates (650, 1,300, 2,600, and 3,300 sec^{-1}) and exposure times. At no condition was a significant decrease in C + S ever observed (at 1,300 sec^{-1} and three minutes it was somewhat increased). In contrast, adhesion was decreased at all shear rates studied (650 to

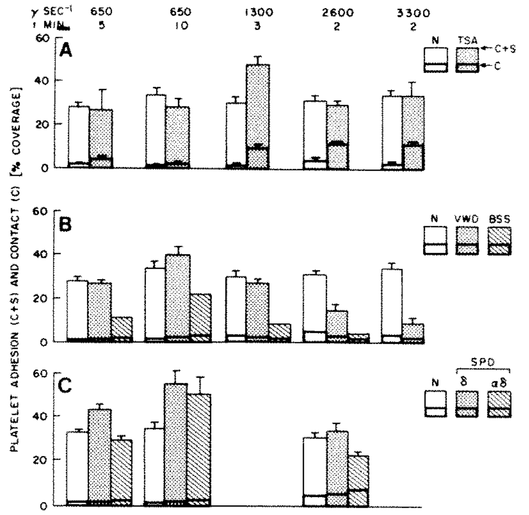

Fig 1. Platelet interation with subendothelium in nonanticoagulated blood. Venous blood was drawn at varying shear rates (γ) and perfusion times (*t*) directly through the annular perfusion chamber containing everted de-endothelialized vessel segments, and the percentage of the surface covered with C or S platelets was determined. Platelet adhesion is defined as C + S. Results (mean ± SE) obtained in normal subjects are compared with (A) thrombasthenia (TSA), (B) severe von Willebrand's disease (vWD) and the Bernard-Soulier syndrome (BSS), and (C) patients (albinos and non-albinos) with SPD who are selectively deficient in dense granules (δ-SPD) and those (family C and patient J.C.) who have deficiencies in both α and dense granules (αδ-SPD).

2,600 sec^{-1}) in patients with the Bernard-Soulier syndrome and was decreased in a shear-rate–dependent manner in von Willebrand's disease (Fig 1, middle). Although the C + S values were normal in thrombasthenia, the number of platelets that had contacted the subendothelium without spreading was consistently increased (Fig 1, top), indicating that thrombasthenic platelets in nonanticoagulated blood do not spread normally on this surface.

Storage pool deficiency. Studies in SPD were performed at shear rates of 650 sec^{-1} (five- and ten-minute perfusion) and 2,600 sec^{-1} (two-minute perfusion). Values for C + S, thrombus volume, and thrombus height obtained in subgroups of δ-SPD (A and NA) and αδ-SPD (family C, and patient J.C.) are shown in Table 2. The pooled C + S and S values for the two types of granule defects (δ- and αδ-SPD) are shown n Fig 1 (bottom) for comparison with the other platelet disorders depicted in that Figure.

Adhesion values (Fig 1, bottom) were somewhat dependent on the shear rate. At 650 sec^{-1}, values in δ-SPD were increased (five- and ten-minute perfusion) and in αδ-SPD were either normal (five-minute perfusion) or increased (ten-minute perfusion). At 2,600 sec^{-1}, values were normal in δ-SPD, but decreased in αδ-SPD (Fig 1, bottom, and Table 2).

The most striking impairment observed was in platelet thrombus dimensions (thrombus height and thrombus volume), and the abnormalities were related to the type of SPD. Among patients with δ-SPD, thrombus dimensions (thrombus height in particular) were more strikingly decreased in albino patients, δ-SPD(A), having severe dense granule defects than in the nonalbino patients with δ-SPD(NA) (Table 2). Thrombus dimensions were decreased in general, to an even greater extent in αδ-SPD (Table 2). The greatly diminished values of thrombus volume and thrombus height in αδ-SPD were particularly evident at 2,600 sec^{-1} in patient J.C. with the most severe α-granule defect.

Studies Using Citrated Blood

Studies using citrated blood were performed at shear rates of 800 and 2,600 sec^{-1} and a perfusion time of five minutes. As in previous studies,[3,29] the platelet thrombus heights (14 ± 2 and 14 ± 4 μm) and thrombus volumes (0.81 ± 0.27 and 0.82 ± 0.30 μm^3/μm^2) in normal subjects were considerably less than the values obtained (Table 2) for nonanticoagulated blood.

Table 2. Platelet-Subendothelial Interaction in SPD in Nonanticoagulated Blood

	Platelet Adhesion (C + S) (Percent Surface Coverage)			Thrombus Volume (μm³/μm²)			Maximum Thrombus Height (μm)		
Shear Rate (sec⁻¹)	650		2,600	650		2,600	650		2,600
Exposure Time (min)	5	10	2	5	10	2	5	10	2
Normal subjects (N = 19, 13, 16)*	27 ± 2	34 ± 3	32 ± 2	1.8 ± 0.3	8.1 ± 0.9	3.4 ± 0.4	76 ± 9	151 ± 14	62 ± 4
SPD									
δ-SPD									
A (N = 4, 4, 4)	47 ± 2†	59 ± 9‡	38 ± 4	1.6 ± 0.2	3.5 ± 0.6§	1.0 ± 0.4§	33 ± 2§	46 ± 5†	16 ± 3†
NA (N = 2, 2, 3)	36	47	29 ± 4	1.5	5.7	1.8 ± 0.5	44	77	41 ± 9‖
αδ-SPD									
Family C (N = 3, 3, 3)	27 ± 1	41 ± 2	24 ± 1§	0.5 ± 0.1†	2.2 ± 0.6‡	0.6 ± 0.3‡	21 ± 2§	38 ± 2§	17 ± 3†
Patient J.C.	37	75¶	20	1.2	1.9	0.07¶	39	32¶	4¶

*No. of subjects for the three flow conditions shown.

†$P < .001$ relative to normal subjects.

‡$P < .01$ relative to normal subjects.

§$P < .05$ relative to normal subjects.

‖$P < .05$ relative to albino subjects with δ-SPD(A).

¶Greater than 2 SD from the mean value of normal subjects.

Values shown are the mean ± SE for N ≥ 3, average for N = 2, or single value for patient J.C.

GPIIb-IIIa deficiency. In contrast to the normal values observed in nonanticoagulated blood, C + S in citrated blood was decreased in thrombasthenia by an average of 50% and 59%, respectively, at both 800 and 2,600 sec^{-1} (Fig 2), but no increase in contact platelets was observed. The magnitude of the adhesion defect was less than in the Bernard-Soulier syndrome (Fig 2) but was similar to that obtained in patients with severe von Willebrand's disease under the same conditions (Fig 2). In the latter disorder, however, the decreased adhesion (33% and 68% reduction at 800 and 2,600 sec^{-1}) was more shear-rate dependent (Fig 2). Addition of a monoclonal antibody against GPIIb-IIIa to normal blood completely eliminated platelet-platelet interaction (absent thrombi) on the subenbdothelium when evaluated after perfusion for five minutes at a shear rate of 2,600 sec^{-1} and, hence, produced the type of defect observed in thrombasthenia (data not shown). This antibody reduced platelet adhesion at a shear rate of 2,600 sec^{-1} to a level (Fig 3) that was comparable to the values observed in thrombasthenia. In contrast, no inhibition of adhesion was observed at a shear rate of 200 sec^{-1} (Fig 3).

Storage pool deficiency. In $\alpha\delta$-SPD, adhesion was decreased significantly ($P < .01$) at 800 sec^{-1} and almost significantly ($P = .07$) at 2,600 sec^{-1} (patient J.C., with the most severe defect, was not, unfortunately, studied under these conditions).

In δ-SPD, adhesion values at 800 sec^{-1} were virtually identical to those obtained in normal subjects (Fig 2). At 2,600 sec^{-1}, the value (30.2% ± 7.1%) was 33% less than normal (44.7% ± 4.4%), but this reduction failed to reach statistical significance at the 5% level ($P = .08$). Since considerable variation was obtained among subjects in this group of nine patients with δ-SPD, we examined other parameters that could have influenced adhesion values at 2,600/sec^{-1}. Among several parameters that could have influenced adhesion, we found no correlation with the platelet count or plasma VIIIR:Ag value. However, a strong correlation ($r = .88$, $P < .01$) with the hematocrit value was observed, whereas no correlation ($r = .27$) was observed in normal subjects. In addition, as seen in Fig 4, when patients and control subjects were each subdivided into two groups (one with hematocrit values 40 or less and the other with values greater than 40), it could be seen that the decreased adhesion in δ-SPD was limited to those five subjects with the lower hematocrits. In these patients (hematocrit value, 38.4 ± 0.7) the adhesion value was 17.7% ± 3.7%, compared with a value of 44.7% ± 4.6% ($P < .01$) in the subset of control subjects with the comparable hematocrits (39.0 ± 0.6). In contrast, the average adhesion value (Fig 4) in the four patients with higher hematocrit values (43.0 ± 0.4) were not significantly different than in controls with comparable hematocrit values (44.0 ± 0.5).

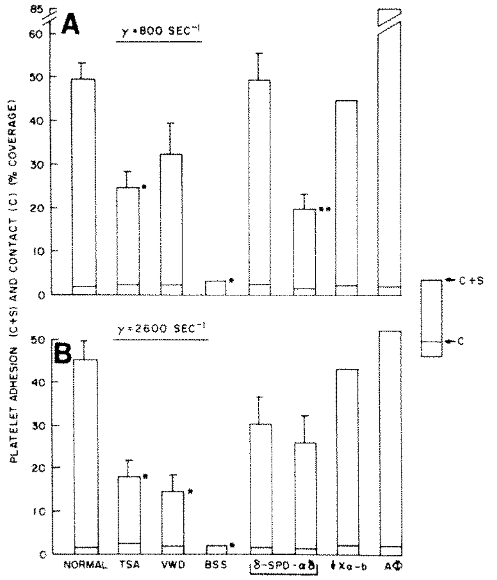

Fig 2. Platelet interaction with subendothelium in citrated blood. The everted rabbit segments were exposed to citrated blood for five minutes at shear rates (γ) of 2,600 sec^{-1} (B) and 800 sec^{-1} (A). Values for C + S and C are shown for normal subjects and in patients with thrombasthenia, ($n = 5$), severe von Willebrand's disease ($n = 3$), the Bernard-Soulier syndrome ($n = 2$), SPD with dense granule defects only (δ-SPD, $n = 9$), SPD with combined deficiencies of α and dense granules ($\alpha\delta$-SPD, family C, $n = 3$), Scott syndrome (decreased X_a binding, $n = 1$), and severe fibrinogen deficiency (A ϕ, $n = 1$). Values are the mean ± SE. *P* values (relative to normal subjects) are indicated as * and ** for .05 and .01 significance levels, respectively.

Other subjects. In the patient with severe fibrinogen deficiency, platelet adhesion was either normal (2,600 sec^{-1}) or somewhat increased (800 sec^{-1}). Adhesion was normal at both shear rates in the patient with Scott syndrome (Xa binding defect) (Fig 2).

DISCUSSION

In a previous study, we have described and formally analyzed a model for platelet adhesion on subendothelium in our perfusion system and shown how defects in either initial platelet contact or subsequent platelets spreading could effect the measured values of C, S, and C + S (defined as total adhesion) as a function of time.[4] From the results of that study, we concluded that GPIb plays a role in platelet adhesion by mediating the initial contact of platelets with the subendothelial surface. A major purpose of the present study was to evaluate a possible role for GPIIb-IIIa in mediating platelet adhesion to subendothelium by studying platelets deficient in these glycoproteins. In addition, we used patients with SPD to evaluate whether granule-bound substances might also mediate adhesion and thrombus formation.

In studies using nonanticoagulated blood, we identified several properties of platelets that influence platelet-platelet interaction (thrombi) on subendothelium. As previously observed with citrated blood,[41] platelet thrombi were completely absent in thrombasthenia, indicating that GPIIb-IIIa is required for platelet-platelet interaction. Thrombi were also smaller (but not absent) in patients selectively deficient in dense granules (δ-SPD), and the degree of abnormality was in proportion to the magnitude of the δ-SPD (Table 2). These findings strongly suggest that dense granule–bound substances, in all likelihood ADP, either influence the growth of thrombi on subendothelium or help to maintain thrombus stability in growing thrombi subjected to the

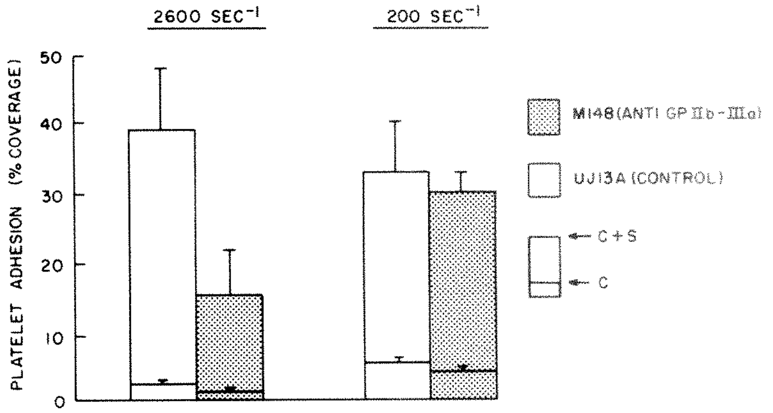

Fig 3. Effect of a monoclonal antibody to GPIIb-IIIa on platelet adhesion. A monoclonal antibody to GP IIb-IIIa (M148) was incubated with normal citrated blood (n = 4 subjects) for 30 minutes at 37 °C with a final concentration of 10 μg/mL. The blood was then circulated through the perfusion chamber containing the vessel segments for five minutes at shear rates of 200 or 2,600 sec^{-1}, and both platelet C and C + S were determined. Studies were also obtained by similarly evaluating blood (from the same four subjects) that had been incubated with a control antibody (UJ13A) at the same concentration. C + S values are the mean ± SE.

high-shear stresses (650 to 2,600 sec^{-1} in the present study) comparable to those encountered physiologically.[42] The even smaller thrombi (Table 2) observed in patients with combined deficiencies of α and dense granules ($\alpha\delta$-SPD) further suggest an additional role for substances, including vWF,[31] stored in α-granules as well.

Using both nonanticoagulated and citrated blood, we observed abnormalities in the interaction of platelets with the subendothelium that also indicate a possible role for GPIIb-IIIa in mediating platelet adhesion. In nonanticoagulated blood, we found that the number of C platelets in thrombasthenia was consistently higher than in normal blood, even where C + S values were comparable in the two groups (Fig 1), indicating impaired spreading of thrombasthenic platelets on the subendothelial surface. In citrated blood (Fig 2), the 50% to 59% reduction in C + S values were comparable to those observed in von Willebrand's disease (although the abnormality in the latter disorder was somewhat more shear dependent, suggesting different mechanisms for the defect). In addition, when GPIIb-IIIa in normal platelets was blocked by a monoclonal antibody to GPIIb-IIIa, the resulting reduction in platelet adhesion was comparable to that observed in thrombasthenia (Fig 3). The decreased values for C + S that we obtained in the present study in the thrombasthenic patients are at some variance with the conclusions of a previous study using citrated blood and a shear rate of 800 sec^{-1}.[41] However, these earlier studies were conducted at longer perfusion times for which the surface in normal controls approaches surface saturation with platelets, so that potential differences between patients and controls are reduced. The values obtained at the shorter perfusion times of the present experiments are not in the saturation region and, hence, would be more likely to demonstrate differences between patients and controls. In fact, the results are quite compatible with the originally reported patient results for longer perfusion times, which were at the lower limit of normal even in that study.

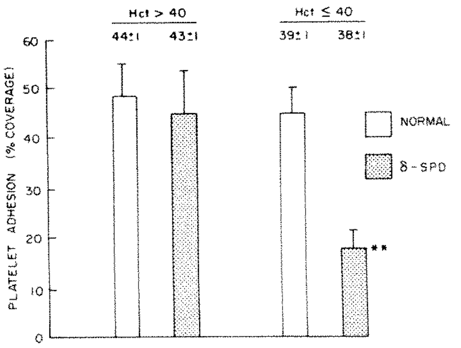

Fig 4. Effect of hematocrit value on platelet adhesion in δ-SPD. C + S values on subendothelium are shown for normal control subjects (n = 17) and patients with isolated dense granule defects, δ-SPD, (n = 9) who were subdivided into two sets of groups. In one set (left), all controls (n = 9) and patients (n = 4) had hematocrit values greater than 40, and in the other set (right), all controls (n = 8) and patients (n = 5) had values 40 or less. Note that average hematocrit (Hct) values for controls and patients within these two subgroups were similar. The symbol ** denotes that, in the lower hematrocrit value groups, the difference between patients and controls was significant, $P < .01$. For the nine patients with δ-SPD, the correlation ($r = .88$) between hematrocrit and adhesion values was also highly significant ($P < .01$). Studies were performed at a shear rate of 2,600 sec^{-1} and a perfusion time of 5 min.

The results of our study as well as those of a recent report demonstrating a requirement for GPIIb in mediating platelet-collagen adhesion[43] indicate that the GPIIb-IIIa complex may be considered an adhesion site on the platelet surface. As previously indicated, the process by which platelets (and other cells[1]) adhere to a surface consists of an initial attachment phase (which we denote as C platelets in our morphometric analysis) and a subsequent spreading (measured as S platelets) on the subendothelium. We believe that the results obtained with nonanticoagulated blood (increased values for C, but normal C + S) and citrated blood (decreased C + S values) ccmplement each other in suggesting an abnormality in platelet spreading. In a previous paper, we analyzed, on theoretical grounds, how defects in either platelet C or S will effect the values for C + S as a function of both perfusion time and another critical variable, the rate (K_d) at which platelets detach from the surface.[4] The model predicted that under conditions in which the rate of detachment was low, the major effect of a platelet spreading defect would be consistently higher values of C at all perfusion times, with relatively unaffected values for C + S. The values we obtained in nonanticoagulated blood are consistent with such a defect. In contrast, an increased detachment rate would produce conditions that are more sensitive for detecting a defect in C + S, but relatively less sensitive for detecting an increase in platelet C, as observed in our studies on citrated blood. Although we have no direct data to suggest that removal of calcium is more favorable for the detachment of contacted platelets, such a mechanism might explain how a defect in platelet spreading (because of a GPIIb-IIIa deficiency) could result in increased C values in nonanticoagulated blood and decreased C + S values in citrated blood.

Another possible reason why a reduction in C + S in thrombasthenia is observed in citrated but not nonanticoagulated blood is related to potential effects of platelet thrombus size in influencing adhesion. In previous studies,[3,29] we have shown that the presence of large thrombi tend to reduce platelet adhesion on the surface. Two potential mechanisms may contribute to the reduced adhesion in the presence of thrombus growth, especially in nonanticoagulated blood in which thrombus heights reach 100 to 200 μm, (in citrated blood, thrombus heights rarely exceed 30 μm). One possibility is that platelets near the surface of growing thrombi are depleted in number by being adsorbed by the thrombus mass. The other possibility is that thrombi shelter the surface downstream of the growing thrombus from circulating platelets. Both these mechanisms contribute to the localized depletion of the platelet boundary layer concentration; moreover, the larger the thrombus growth, the greater the depletion. Therefore, the smaller thrombi observed in patients with SPD (Table 2 and Fig 2) could explain the relatively higher C + S values obtained at some conditions in these patients. The aforementioned observations would predict an even greater enhancement of adhesion in thrombasthenia, where thrombi are completely absent, than in SPD. That adhesion was generally normal could, therefore, reflect the combined effects of absent thrombi (tending to increase adhesion) and a true adhesion defect (tending to reduce it). In contrast, thrombi in citrated blood are considerably smaller than in nonanticoagulated blood, thereby reducing the influence of tall thrombi on adhesion and enhancing the possibility of observing decreased C + S values in thrombasthenia.

The mechanism by which GPIIb-IIIa mediates platelet spreading and adhesion on subendothelium remains to be determined. Since adhesion was not decreased (and was, in fact, increased) in a patient with severe fibrinogen deficiency, it is unlikely that the binding of fibrinogen to GPIIb-IIIa plays a role. The recent observations that thrombin and ADP can promote the binding of vWF to platelets[16,18-22] through GPIIb-IIIa[16,20,22] suggest that this protein, in addition to supporting adhesion through binding to GPIb, may also mediate platelet–vessel wall attachment through binding to GPIIb-IIIa. The decreased adhesion in some patients with δ-SPD (as in fawn-hooded rats with SPD[44]) is also consistent with the recent conclusions of Timmons et al that released platelet ADP could also play a role in this mechanism.[23] However, platelet adhesion in δ-SPD was strongly influenced (at 2,600 $\sec^{-1}$) by the hematocrit value, even within the relatively narrow range of hematocrit values (36 to 44) observed in the study (Fig 4). These findings suggest a possible role for red cells that could bypass the platelet contribution with increasing shear rates, as was previously suggested.[45] This possibility is currently under investigation.

The question whether the aforementioned conclusions are valid must take into account the recent studies that have shown that GPIIb-IIIa–mediated binding of exogenously added vWF does not occur in the presence of physiologic concentrations of fibrinogen.[24,25] However, this would not necessarily apply to vWF present in platelet α-granules and secreted at the platelet surface following platelet activation. Previous studies, for example, have shown a higher concentration of high–molecular weight multimers of vWF in platelets than in plasma,[35,46] and it is possible that platelet vWF could have a higher affinity for GPIIb-IIIa than plasma vWF. In addition, whereas the molar ratio of fibrinogen (10^{-5} mol/L) to vWF (5×10^{-8} mol/L for a subunit molecular weight (mol wt) of 220,000[24]) in plasma is about 200, the ratio in platelets is approximately 14 (assuming a fibrinogen concentration of approximately 100 μg/10^9 platelets,[35] a VIIIR:Ag concentration of 0.43 U/10^9 platelets,[34] and an assumption that, as in plasma, 1 unit of VIIIR:Ag per ml is equal to 5×10^{-8} mol/L for a subunit mol wt of 220,000). In addition, vWF in subendothelium has also been demonstrated to play a role in mediating platelet adhesion,[5] and it is possible that vWF bound to a surface may more successfully compete with fibrinogen for GPIIb-IIIa on platelets that have initially attached through GPIb[4] than does plasma vWF. Finally, α-granule proteins other than vWF (such as fibronectin and thrombospondin) could also play a role in mediating platelet adhesion through their binding to GPIIb-IIIa,[47] and the reduced levels of platelet adhesion that we observed in patients with $\alpha\delta$-SPD do not, at present, permit us to choose among several α-granule proteins that could be important. Further studies on the complex interaction of vWF, membrane glycoproteins, and various granule substances could have important implications for understanding hemostasis and thrombosis.

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Excess Release of Ferriheme in G6PD-Deficient Erythrocytes: Possible Cause of Hemolysis and Resistance to Malaria

By Stephen K. Janney, J. Heinrich Joist, and Coy D. Fitch

Hemoglobin in glucose-6-phosphate dehydrogenase (G6PD)-deficient erythrocytes is abnormally vulnerable to oxidative denaturation, which may release ferriheme, a known cytolytic agent. We found 13.3 nmol of ferriheme in G6PD-deficient erythrocyte membranes (per gram of total erythrocyte hemoglobin) using a spectrophotometric assay, as compared to 9.8 in normal membranes ($P < .05$). After incubation of erythrocytes with 250 µmol/L menadione, an oxidant drug, the values increased by 37.4 nmol in G6PD-deficient membranes and by 26 in normal membranes ($P < .005$), indicating increased hemoglobin denaturation. To verify that hemoglobin denaturation in G6PD-deficient erythrocytes releases ferriheme in a form available to interact with other ligands, [^{14}C]-chloroquine binding to intact erythrocytes was measured. With an initial concentration of 5 µmol/L chloroquine in a medium containing no menadione, an excess of 14.8 nmol of chloroquine was bound in G6PD-deficient erythrocytes (per gram of hemoglobin) as compared to normal erythrocytes ($P < .005$). In the presence of 250 µmol/L menadione, chloroquine binding increased by 17.9 nmol in G6PD-deficient and by 7.2 in normal erythrocytes ($P < .005$). These results indicate that ferriheme becomes available to interact with endogenous ligands and, thus, to mediate menadione-induced hemolysis in patients with G6PD deficiency. Furthermore, the increase in ferriheme may mediate the selective toxicity of menadione for *Plasmodium falciparum* parasites growing in G6PD-deficient erythrocytes. Ferriheme release in response to the intraerythrocytic oxidant stress introduced by malaria parasites also may account for the resistance to malaria afforded by G6PD deficiency.

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WHEN *PLASMODIUM FALCIPARUM* infects glucose-6-phosphate dehydrogenase (G6PD)-deficient erythrocytes, parasite maturation is delayed and impaired.[1-4] Presumably, the oxidant stress introduced by the parasite is sufficient to overwhelm the antioxidant capacity of G6PD-deficient erythrocytes, leading to the accumulation of free radicals and other toxic substances.[2,5,6] Ferriheme may be one of these toxic substances. Hemoglobin in G6PD-deficient erythrocytes undergoes denaturation during oxidant stress,[7-10] oxidative denaturation may release ferriheme,[8,10,11] and ferriheme is a potent lytic agent for erythrocytes and malaria parasites.[12-15] The term ferriheme is used to identify iron(III) protoporphyrin IX. Both hemin, which is iron(III) protoporphyrin IX chloride, and hematin, which is iron(III) protoporphyrin IX hydroxide, are potent hemolytic agents.

In this study, we examined ferriheme release in erythrocytes in the presence or absence of the oxidant drug, menadione. This drug precipitates hemolytic episodes in patients with G6PD deficiency,[16] and it accentuates the inhibition of *P falciparum* parasite development in G6PD-deficient erythrocytes.[2] Moreover, high concentrations of menadione release sufficient amounts of ferriheme in normal erythrocytes to cause hemolysis.[13] When ferriheme is released intracellularly or added exogenously, it is available to interact with cellular constituents, to lyse erythrocytes, and to bind chloroquine with high affinity (K_d of 10^{-6} to 10^{-7} mol/L).[13,17] In mature mammalian erythrocytes, chloroquine does not bind with high affinity unless ferriheme has been released from hemoglobin.[13,17,18] Consequently, it is possible to estimate ferriheme release in intact erythrocytes by measuring chloroquine binding and correcting for low-affinity binding to normal erythrocytic constituents.[13,18]

MATERIALS AND METHODS

Blood was withdrawn by venipuncture from black male patients with G6PD deficiency who were hospitalized at the John Cochran Veterans Administration Medical Center after informed consent was obtained from them. Healthy adult male volunteers provided normal blood. An aliquot of blood was anticoagulated with EDTA and used for cellulose acetate hemoglobin electrophoresis[19] to exclude common hemoglobinopathies and for G6PD measurements.[20] The remainder of the blood was immediately mixed with an equal volume of tris(hydroxymethyl)aminomethane (Tris) medium (141 mmol/L NaCl, 20 mmol/L glucose, and 10 mmol/L Tris, pH 7.4) containing 175 U/mL of heparin. Erythrocytes were sedimented by centrifugation and washed three times with Tris medium to remove plasma and buffy coat before preparing suspensions for incubation with or without menadione and chloroquine, alone or in combination. Prior to incubation, the hemoglobin concentrations of these erythrocyte suspensions were measured by the cyanmethemoglobin method[21] to serve as a common reference for expression of results. Incubations were carried out for 60 minutes at 37 °C using 12.5% (by volume) suspensions of erythrocytes in Tris medium in the absence (control) or presence of menadione. A stock suspension of 5 mmol/L menadione in Tris medium containing 0.08% Tween 80 (Sigma, St. Louis) was prepared and sonicated immediately prior to dilution in the incubation mixture.

To measure membrane ferriheme content, erythrocytes were hemolyzed using 30 vol of a hypotonic medium (5 mmol/L NaCl, 10 mmol/L Tris, pH 8.0) at 4 °C. The membranes were sedimented by centrifugation at 20,000 g for 20 minutes at 4 °C and washed five times with the hypotonic medium.[22] Ferriheme bound to membranes prepared in this way has the spectral characteristics of hemichrome

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and has been used as an indicator of hemoglobin denaturation.[23,24] The ferriheme content of the membranes was measured spectrophotometrically after dissolving them in 2.5% sodium dodecyl sulfate (SDS).[23] This method measures total ferriheme, including any heme present in denaturated hemoglobin (eg, Heinz bodies) or hemichrome bound to or trapped in these washed membrane preparations.

As in previous studies, chloroquine binding to washed, intact erythrocytes was measured after incubation with ring-labeled 3-[^{14}C]-chloroquine (2.36 μCi/μmol; New England Nuclear, Boston).[13,18] The erythrocytes were separated from the medium by centrifugation, and the chloroquine content of each was determined radiochemically after quantitative extraction into heptane under alkaline conditions.[18] The initial concentration of chloroquine in the incubation medium (5 μmol/L) was chosen because ferriheme released intracellularly from hemoglobin binds chloroquine with a dissociation constant of approximately 10^{-6}mol/L.[13] Normal erythrocytes bind chloroquine with low affinity (dissociation constant of approximately 10^{-3} mol/L)[18], attributable to other erythrocyte components such as hemoglobin and methemoglobin.

RESULTS

Under control conditions, the ferriheme content measured spectrophotometrically was greater in isolated membranes from G6PD-deficient erythrocytes than from normal erythrocytes (Fig 1). The values were 13.3 ± 2.8 (mean ± 1 SD) nmol of membrane ferriheme per gram of erythrocyte hemoglobin for G6PD-deficient and 9.8 ± 2.4 for normal membranes ($P < .05$, Student's *t* test). After incubation with 250 μmol/L menadione, these values increased by 37.4 to 50.7 ± 5.9 for G6PD-deficient and by 26 to 35.8 ± 3.9 for normal membranes ($P < .001$). Since the spectrophotometric method measures total ferriheme, these results only confirm that menadione denatures intracellular hemoglobin[25]; they do not provide quantitative information about the concomitant release of ferriheme in a form available to interact with erythrocytic constituents.

To evaluate ferriheme release, chloroquine binding to intact erythrocytes was measured (Fig 1). Under control conditions, 69.5 ± 8.6 nmol of chloroquine were bound per gram of hemoglobin in G6PD-deficient erythrocytes and 54.7 ± 4.0 nmol were bound in normal erythrocytes ($P < .005$). After incubation with 250 μmol/L menadione, these values increased by 17.9 to 87.4 ± 6.1 in G6PD-deficient and by 7.2 to 61.9 ± 3.4 in normal erythrocytes ($P < .005$). These increases in chloroquine binding correlate with the measured increases in ferriheme content of erythrocyte membranes (Fig 2).

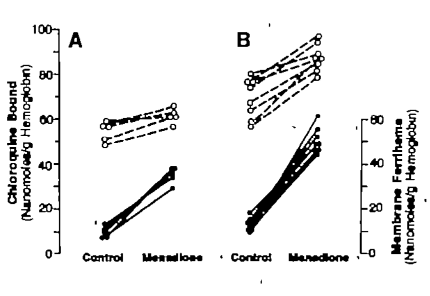

Fig 1. Effect of menadione on chloroquine binding to erythrocytes and on ferriheme content of erythrocyte membranes. Suspensions of erythrocytes were incubated in the absence (control) or presence of 250 μmol/L menadione. Chloroquine binding to intact erythrocytes and membrane ferriheme content were measured as described in the text. The values shown are the results of separate experiments with erythrocytes obtained from five normal subjects (A) and eight patients with G6PD deficiency (B). Key: ○, chloroquine bound to intact erythrocytes; ●, ferriheme content of isolated membranes.

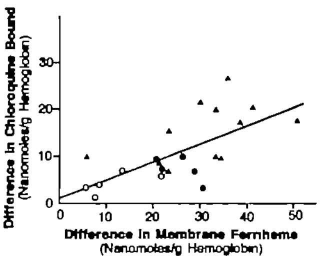

Fig 2. Comparison of chloroquine binding to intact erythrocytes with ferriheme content of isolated erythrocyte membranes. Chloroquine binding (difference between the values in the presence and absence of menadione) is plotted on the ordinate; ferriheme content (difference between the values in the presence and absence of menadione) is plotted on the abscissa. The results shown are from the 13 experiments reported in Fig. 1 and an additional nine experiments carried out in an identical manner except that the menadione concentration was 125 μmol/L. Key: ○, normal erythrocytes with 125 μmol/L menadione; △, G6PD-deficient erythrocytes with 125 μmol/L menadione; ●, normal erythrocytes with 250 μmol/L menadione; ▲, G6PD-deficient erythrocytes with 250 μmol/L menadione. The regression line ($Y = 0.38X + 1.18$) is shown; the correlation coefficient is .666 ($P < .01$).

DISCUSSION

These results indicate that there is excess release of ferriheme in G6PD-deficient erythrocytes and that oxidant stress further increases this excess. To use chloroquine binding to estimate the amount of ferriheme available to interact with endogenous ligands, it is necessary first to correct for low-affinity binding to normal erythrocytic constituents.[13] The magnitude of this correction is approximated by the binding of chloroquine to normal erythrocytes under control conditions since normal erythrocytes bind little if any chloroquine with high affinity.[13,18] Using the value for normal erythrocytes as an approximation of low-affinity binding, G6PD-deficient erythrocytes bound an excess of 14.8 nmol of chloroquine per gram of hemoglobin under control conditions, and this excess increased by 17.9 to 32.8 nmol after the stress of 250 μmol/L menadione. The corresponding excess of chloroquine binding for normal erythrocytes stressed with menadione was 7.2 nmol. Assuming that 1 molecule of chloroquine binds two molecules of ferriheme, as has been determined in equilibrium dialysis experiments,[17] it is possible to calculate that G6PD-deficient erythrocytes stressed with menadione contain 65.6 nmol of ferriheme per gram of erythrocyte hemoglobin in a form available to interact with endogenous ligands. The corresponding value for normal erythrocytes stressed with menadione is 14.4.

Since oxidatively stressed G6PD-deficient erythrocytes release an excess of ferriheme, we conclude that ferriheme is available to mediate the hemolysis induced by menadione in G6PD-deficient patients[16] and the selective toxicity of menadione for malaria parasites growing in G6PD-deficient erythrocytes.[2] Release of ferriheme may also be involved in the pathogenesis of hemolytic episodes caused by other oxidant drugs in G6PD-deficient patients. In addition, G6PD-deficient erythrocytes may be particularly vulnerable to the intraerythrocytic oxidant stress produced by malaria parasites, which metabolize NADPH[26] and generate H_2O_2.[5] The resultant release of ferriheme could inhibit intraerythrocytic development of malaria parasites in G6PD-deficient erythrocytes, providing an explanation for the mechanism of reduced parasitemia that has been observed, and thus could explain genetic selection for G6PD deficiency.

Even in the absence of abnormal oxidant stress from drugs or malaria parasites, enough ferriheme may eventually accumulate within some erythrocytes to cause their destruction. Thus, an excess of ferriheme could have contributed to the decreased erythrocyte survival observed by Brewer and associates in black individuals with G6PD deficiency who were not in hemolytic crisis.[27] Furthermore, since there is evidence of a decrease in G6PD activity with increasing age of normal erythrocytes,[28] it is possible that ferriheme accumulation may contribute to the destruction of senescent erythrocytes.

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Inhibition of Neutrophil Oxidative Metabolism by Lysosomotropic Weak Bases

By Barbara Styrt and Mark S. Klempner

Maintenance of an acidic intralysosomal compartment may be relevant to multiple aspects of neutrophil function. The effect of lysosomal alkalinization on the neutrophil respiratory burst was studied by measuring cytochrome c reduction in response to soluble stimuli in the presence of lysosomotropic weak bases. The weak bases chloroquine, ammonium chloride, methylamine, and clindamycin all raised the intralysosomal pH and inhibited neutrophil oxidative metabolism at concentrations ranging from 0.1 to 100 mmol/L. Inhibition was dose dependent for each base and correlated significantly with the degree of lysosomal alkalinization. Concentrations that did not alkalinize the lysosome did not inhibit the respiratory burst. Inhibition by weak bases was seen when oxidative metabolism was stimulated by phorbol myristate acetate, calcium ionophore A23187, formyl-methionyl-leucyl-phenylalanine, opsonized zymosan, or sodium fluoride. Increasing the stimulus concentration (from 5 ng/mL to 5 µg/mL phorbol myristate acetate and from 0.5 to 1 µmol/L A23187) diminished or abolished inhibition by weak bases. Washing the cells after incubation with bases and before stimulation substantially reversed the inhibition. None of the bases impaired detection of superoxide in a cell-free xanthine-xanthine oxidase assay. Other indexes of oxidative metabolism, including oxygen consumption and hydrogen peroxide release, were also inhibited by weak bases. Analysis of particulate NADPH oxidase activity from neutrophils stimulated in the presence of bases suggested that these cells assemble a subnormal amount of an enzyme complex with normal kinetic characteristics. Lysosomotropic weak bases alkalinized the neutrophil lysosome and produced inhibition of oxidative metabolism that was dose related, was not stimulus specific, and was largely reversed by washing the cells before stimulation. A possible explanation would be altered assembly of the enzyme complex involved in respiratory burst activation as a consequence of impaired granule/plasma membrane fusion in the presence of diminished transmembrane pH gradients.

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THE HUMAN NEUTROPHIL responds to a variety of stimuli, including phagocytosable particles and membrane-active soluble agents, with a complex sequence of events including degranulation and production of toxic oxygen metabolites. There is evidence to suggest that these two components of the activation response share a requirement for membrane fusion between the neutrophil granules and plasma membrane. In other cell systems, vesicular acidity is thought to be important to reactions between membranes,[1,2] and we have previously demonstrated that weak bases that raise the pH of the acidic neutrophil lysosome are inhibitors of degranulation.[3] To see whether this effect can be generalized to other fusion-requiring elements of the neutrophil activation sequence, we have now examined the effect of weak bases that alkalinize the intralysosomal pH on the neutrophil respiratory burst.

MATERIALS AND METHODS

Hypaque (diatrizoate meglumine and diatrizoate sodium) was obtained from Winthrop Laboratories (New York), dextran (molecular weight, 200,000 to 300,000) from J.T. Baker Co (Phillipsburg, NJ), and phorbol myristate acetate (PMA) from L.C. Services (Woburn, Mass). Ficoll, 9-aminoacridine, cytochrome C, superoxide dismutase, formyl-methionyl-leucyl-phenylalanine (f-met-leu-phe), cytochalasin B, calcium ionophore A23187, chloroquine, methylamine HCl, xanthine, xanthine oxidase, catalase, scopoletin, and zymosan were obtained from Sigma Chemical Co (St Louis). Clindamycin HCl was from the Upjohn Co (Kalamazoo, Mich) and horseradish peroxidase from Boehringer Mannheim (Indianapolis, Ind). Solutions of weak bases were made up at 100 times the desired concentration, and the pH was adjusted with concentrated HCl or NaOH to produce 0.1 pH or less unit change in the incubation buffer.

Isolation of neutrophils. Heparinized venous blood was obtained from healthy volunteer donors after informed consent was obtained from them. Neutrophils were isolated by Ficoll-Hypaque density gradient centrifugation and dextran sedimentation following the method of Boyum.[4] Residual erythrocytes were removed with three cycles of hypotonic saline lysis, and cells were resuspended in Hanks' balanced salt solution (HBSS). The neutrophil concentration was 10^7/mL for superoxide assays and 10^8/mL for lysosomal pH monitoring. For assays of stimulation, the buffer contained 0.9 mmol/L calcium and 0.9 mmol/L magnesium.

Monitoring of intralysosomal pH. The method for following intralysosomal pH in intact neutrophils has been described previously.[3] Briefly, fluorescence was monitored at 400-nm excitation and 456-nm emission in a solution (3 mL) of 0.05 µmol/L 9-aminoacridine maintained at 37 °C by a circulating water bath. Neutrophils (120 µL at 10^8/mL for a final concentration of 4×10^6/mL) were added, and fluorescence quenching was followed for four minutes as the indicator was taken up into the lysosomes. Weak bases were then added, and displacement of the indicator from the cells, because of lysosomal alkalinization, was monitored as increasing fluorescence. The degree of lysosomal alkalinization was estimated from the percent reversal of initial fluorescence quenching, as correlated with previous observations of the transmembrane pH gradient in isolated lysosomes.[5]

Measurement of neutrophil oxidative metabolism. Neutrophils (5×10^6) were preincubated with weak bases (10 µL at 100 times the desired final concentration) in a total volume of 0.9 mL for five minutes at 37 °C. Cytochrome c (0.1 mL at 0.001 mol/L) was added, followed by the stimulus, and the incubation was continued for an additional 20 minutes. When f-met-leu-phe or opsonized zymosan was used as the stimulus, cells were incubated with cytochalasin B (5 µg/mL) for an additional five minutes before

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stimulation. For all conditions, simultaneous controls contained 0.1 mg/mL superoxide dismutase (SOD).

Incubation was terminated by centrifuging the samples for ten minutes at 1,200 rpm and 4 °C. Absorbance of supernatants (diluted 1 in 4 in 0.1 mol/L phosphate buffer, pH 7.2) was scanned from 535 to 555 nm in a Gilford model 250 spectrophotometer (Gilford Instrument Laboratories, Oberlin, Ohio), either immediately or after storage at −70 °C. The optical density $(OD)_{550-540}$ was compared in duplicate samples with and without SOD, and superoxide production was calculated as the SOD-inhibitable reduction of cytochrome c using an extinction coefficient of 21.1/mmol/L·cm.[6]

The effect of weak bases was calculated as the decrease in cytochrome c reduction by neutrophils incubated with weak bases relative to control cells from the same donor processed identically except for the absence of the base. Results are expressed as percent inhibition (mean ± SEM of two to three determinations).

Reversibility. To document whether the effect of weak bases was reversible, neutrophils were preincubated with 1 mmol/L chloroquine or 50 mmol/L NH_4Cl for ten minutes at 37 °C, centrifuged for ten minutes at 1,200 rpm at 4 °C, washed once with HBSS, and resuspended in HBSS with divalent cations. Superoxide production was then measured as previously outlined.

Xanthine-xanthine oxidase assay. Superoxide scavenging by weak bases was determined by using a cell-free enzyme system to generate superoxide.[7] Reaction mixtures contained 33 μmol/L cytochrome c, 100 μmol/L xanthine, and 0.26 U/mL xanthine oxidase in 0.05 mol/L phosphate buffer (pH 7.5) as well as 67 μg/mL SOD and weak bases where indicated. OD_{550} was read after five minutes of incubation at room temperature. SOD-inhibitable cytochrome c reduction was determined for duplicate samples with and without the base, and the percent inhibition by the base was calculated.

Preparation of particulate fraction containing NADPH oxidase activity. To prepare particles containing NADPH oxidase activity from stimulated cells,[8] neutrophils were suspended in 2 mL HBSS at approximately 1 to 1.5 × 10^8/mL. Zymosan (45 mg in 5 mL 1 N NaOH) was boiled for ten minutes, washed twice, opsonized in AB serum for 30 minutes at 37 °C, washed twice, and suspended in 2 mL HBSS with 2 mmol/L NaN_3. Neutrophils and zymosan were incubated for three minutes separately at 37 °C. The two suspensions were combined, and incubation was continued for another seven minutes, then terminated by the addition of 4 mL ice-cold HBSS and centrifugation for 12 minutes at 1,200 rpm and 4 °C. The cells were resuspended in 0.34 mol/L sucrose containing 5 × 10^{-4} mol/L phenyl methyl sulfonyl fluoride and sonicated in an ice bath with an ultrasonic probe (Branson Sonic Power Co, Danbury, Conn) for one minute at a setting of 1.5. Undisrupted cells and zymosan were removed by centrifugation at 1,400 rpm for ten minutes at 4 °C. The supernatant was then ultracentrifuged at 4 °C and 25,000 rpm for 30 minutes. The pellet from this ultracentrifugation was suspended in 5 mL 0.34 mol/L sucrose and stored at −70 °C until used. Protein was determined by the method of Bradford.[9]

NADPH oxidase assay. NADPH oxidase activity in the particulate fraction from stimulated neutrophils was determined by measuring the reduction of cytochrome c by monitoring the OD at a wavelength of 550 at room temperature. The reaction was initiated by adding 0.2 mL particles to a total of 3 mL HBSS containing 0.6 mg/mL cytochrome C, 0.02% Triton X-100 (Sigma Chemical Co, St Louis) and varying concentrations of NADPH. The OD was read every 10 seconds for three minutes, and the rate of cytochrome c reduction was taken as the initial linear rate of change after subtracting out control values from identical reaction mixtures to which SOD (0.1 mg/mL) was added.

Determination of kinetic constants for NADPH oxidase. The kinetic constants K_m (substrate concentration at which the rate is half maximal) and V_{max} (reaction rate when enzyme is saturated) for NADPH oxidase were determined by graphic methods from rates of cytochrome c reduction at NADPH concentrations ranging from 0.01 to 2 mmol/L.[10] A Hewlett-Packard 9845A computer was used to generate Lineweaver-Burk plots using least-squares fitting of lines to the data, and K_m and V_{max} were calculated from the slope and intercept.[11] The effect of lysosomotropic bases on these values was assessed by adding 1 mmol/L chloroquine or 50 mmol/L NH_4Cl at three different time points: before activation of neutrophils with zymosan, seven minutes after activation (with 37 °C incubation continued for an additional five minutes after the addition of bases), and after isolation of particles (bases added to the reaction mixture for the NADPH oxidase assay).

Oxygen consumption. Oxygen consumption was monitored with a Yellow Springs oxygen electrode (Yellow Springs Instrument Co, Yellow Springs, Ohio). Neutrophils were suspended in HBSS at 5 × 10^6/mL and loaded with chloroquine (1 mmol/L) or ammonium chloride (50 mmol/L) for five minutes at 37 °C. Cytochalasin B (5 μg/mL) was added in a second five-minute, 37 °C incubation for experiments using f-met-leu-phe as the stimulus. The remainder of the experiment was carried out at room temperature. One milliliter of cells was added with constant stirring to 1 mL of buffer containing the same concentration of base in the electrode chamber. After the electrode baseline was recorded for three minutes, the stimulus (10 μL of 10^{-5} mol/L f-met-leu-phe or 500 ng/mL PMA) was added and the change in pO_2 followed for ten minutes. This value was converted to an estimate of the quantity of oxygen consumed on the basis of the calculated oxygen content of water in equilibrium with air at room temperature.[12]

Hydrogen peroxide production Neutrophil production of hydrogen peroxide was measured fluorometrically by the scopoletin reduction assay.[13] Neutrophils (10^6/mL) were incubated for five minutes at 37 °C in HBSS containing 4 μmol/L scopoletin and 20 nmol/L horseradish peroxidase with or without 50 mmol/L NH_4Cl. Cytochalasin B (final concentration of 5 μg/mL from stock kept at 1 mg/mL in dimethyl sulfoxide [DMSO]) was added for a further five-minute incubation. Then f-met-leu-phe (final concentration of 10^{-7} mol/L) was added. Lag time was estimated from the continuous chart recording, and the initial rate of H_2O_2 production was determined from the reduction in scopoletin fluorescence (excitation wavelength, 350; emission wavelength, 460) Standards of a known H_2O_2 concentration with and without NH_4Cl were used to convert the results to nanomoles of H_2O_2 produced.

RESULTS

Measurement of lysosomal alkalinization. Release of the indicator 9-aminoacridine from neutrophils by weak bases is shown in Fig 1, which compares the concentration response to ammonium chloride[3] and other bases. The rise in intralysosomal pH can be extrapolated from reversal of quenching, as previously reported.[3] Thus, 20% reversal of quenching corresponds approximately to 0.5, and 30% to 1.5-pH–unit alkalinization. Chloroquine, ammonium chloride, methylamine, and clindamycin all produced substantial alkalinization of the lysosome at concentrations ranging from .01 to 100 mmol/L.

Inhibition of superoxide production. To observe either augmentation or inhibition of the respiratory burst, our initial experiments were designed to produce approximately half-maximal stimulation of neutrophils. This level of stimulation was observed using 5 ng/mL PMA, with minimal superoxide production at 0.5 ng and maximum stimulation at

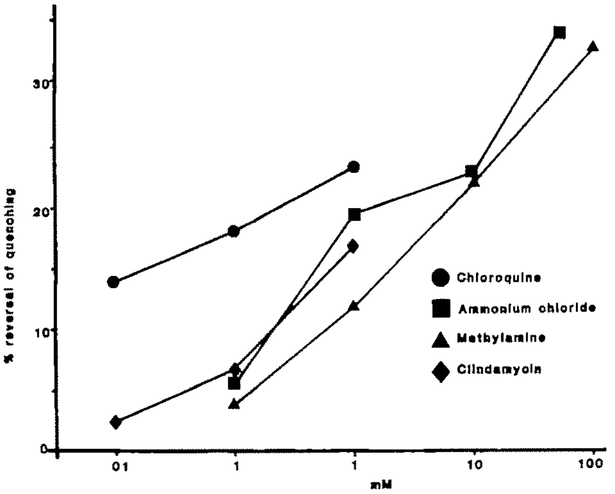

Fig 1. Alkalinization of the neutrophil lysosome by weak bases as monitored by the reversal of 9-aminoacridine fluorescence quenching. Each point represents the mean of two to three determinations.

50 ng and above. The mean superoxide production by normal cells after each of the stimuli used is listed in Table 1.

Figure 2 shows the effect of the four weak bases on superoxide production stimulated by 5 ng/mL PMA. At concentrations that did not alkalinize the lysosome, none of these agents significantly affected oxidative metabolism. At concentrations producing more than 0.5-pH–unit alkalinization, all four bases were inhibitory. Clindamycin inhibition, which we have discussed in detail elsewhere,[14] is here compared with that produced by the other bases. Chloroquine and clindamycin showed somewhat more inhibition relative to their alkalinizing capacity than ammonium chloride and methylamine.

Effect of stimulus concentration. To determine whether the inhibitory effect of weak bases could be overcome, we repeated these experiments using 5 μg/mL PMA for maximal stimulation. As shown in Fig 3, inhibition by 1 mmol/L chloroquine was reduced in this setting. At the highest concentrations of ammonium chloride and methylamine used, inhibition was abolished by raising the stimulus to maximal levels.

Table 1. Relationship Between Neutrophil Response to Different Stimuli and Inhibition of Superoxide Production by 1 mmol/L Chloroquine

Stimulus	Cytochrome c Reduction by Control Cells (nmol)	Percent Inhibition by 1 mmol/L Chloroquine
PMA, 5 μg/mL	67.0 ± 5.1	45.5 ± 10.5
PMA, 5 ng/mL	44.4 ± 6.3	69.5 ± 13.9
NaF, 15 mmol/L	42.1 ± 3.5	96.1 ± 1.5
Opsonized zymosan, 2 mg/mL	33.7 ± 3.7	91.4 ± 7.2
f-met-leu-phe 0 1 μmol/L	30 8 ± 6.9	87.4 ± 6.4
Opsonized zymosan, 0.5 mg/mL	19 3 ± 4.8	89 0 ± 7.1
A23187, 1 μmol/L	13.4 ± 2.5	58.9 ± 19.8
A23187, 0.5 μmol/L	11 2 ± 2 0	90.6 ± 0 3
f-met-leu-phe, 0.01 μmol/L	4.2 ± 2 1	58.4 ± 8.4

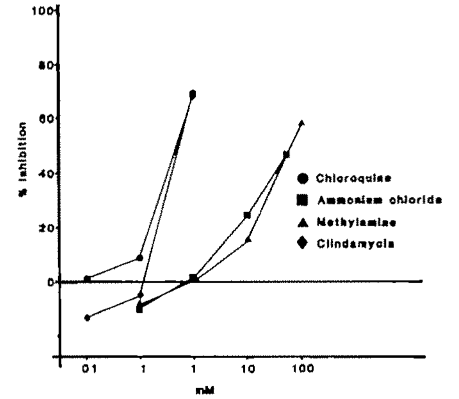

Fig 2. Percent inhibition of PMA-stimulated (5 ng/mL) cytochrome c reduction by varying concentrations of weak bases (mean of two to three experiments).

Assessment of stimulus specificity. To determine whether inhibition by weak bases was stimulus specific, we repeated superoxide assays using other stimuli. Figure 4 shows the effects on superoxide production stimulated by the calcium ionophore A23187 (0.5 μmol/L). Chloroquine, ammonium chloride, and methylamine all produced even more marked inhibition than in the experiments using PMA. A modest reduction in inhibition was produced by increasing the stimulus to 1 μmol/L. As shown in Fig 5, with 0.1 μmol/L f-met-leu-phe as the stimulus in the presence of 5 μg/mL cytochalasin B, results were similar to those seen with 0.5 μmol/L A23187. Zymosan-induced superoxide production was inhibited by chloroquine (Table 1) and ammonium chloride (73.9% ± 7.0% inhibition by 50 mmol/L ammonium chloride with 2 mg/mL zymosan stimulus). Chloroquine also inhibited superoxide production induced by 15 mmol/L fluoride (18.9% ± 21.7% inhibition at 0.01 mmol/L chloroquine, 29.7% ± 11.9% at 0.1 mmol/L, and 96.1% ± 1.5% at 1 mmol/L).

Correlation between lysosomal alkalinization and inhibition of superoxide production. The relationship between lysosomal alkalinization (as percent reversal of quenching) and inhibition of superoxide production by the various bases tested is illustrated in Fig 6 for cells stimulated by 5 ng/mL

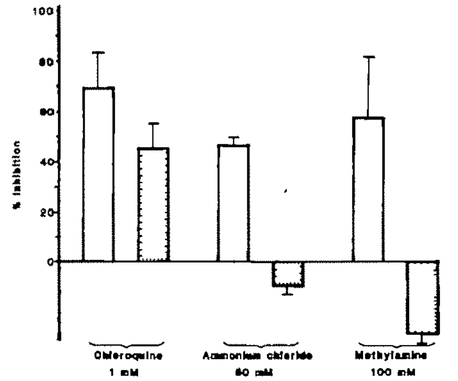

Fig 3. Effect of increased PMA concentration on weak base inhibition of cytochrome c reduction. Each bar represents the mean ± SEM of two to three experiments. ☐, PMA 5 ng/mL; ⊡, PMA 5μg/mL.

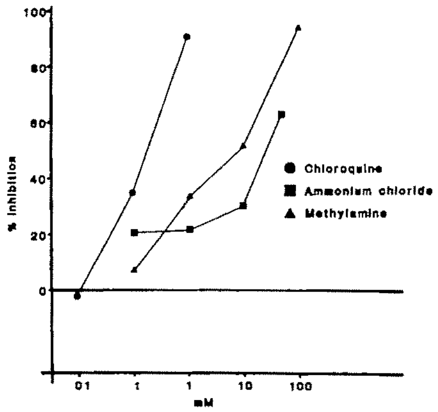

Fig 4. Percent inhibition of A23187-stimulated (0.5 μmol/L) cytochrome c reduction (mean of two to three experiments).

PMA (as in Fig. 2) and by 1 μmol/L A23187. Similar relationships were seen with other stimuli, with correlation coefficients of $r = .904$ ($P < .001$) for 0.1 μmol/L f-met-leu-phe and .752 ($P < .01$) for 0.5 μmol/L A23187. To see whether these correlations were affected by the slight, nonsignificant increase in superoxide production seen with low concentrations of some bases, correlations were also computed omitting negative values for percent inhibition. This procedure reduced r to .548 for cells stimulated with 5 ng/mL PMA but did not significantly weaken any of the other correlations cited.

Although increased concentrations of PMA or A23187 reduced the inhibitory effect of bases on superoxide production, the relationship of greater stimulation to lesser inhibition did not hold when different stimuli were compared. Table 1 shows the amount of superoxide production by control cells (no base) in response to a variety of stimuli. As shown, the ability of chloroquine to inhibit superoxide production was not related to the total amount of superoxide produced.

Reversal of weak base effects. Reversibility of inhibition was assessed by incubating cells with ammonium chloride or chloroquine, washing away the base, and then stimulating the cells with 5 ng/mL PMA. Preliminary experiments had indicated that base-mediated decreases in fluorescence quenching were abolished by this procedure so that the pH gradient across the lysosomal membrane was presumably reestablished at or near its original value before the neutrophils were stimulated. Although slight inhibition was seen after washing, most of the weak base effect on superoxide production was reversed by removal of the bases. Thus, cells treated with 1 mmol/L chloroquine and washed displayed 10.7% ± 8.7% inhibition, compared with 69.5% ± 13.9% with base present throughout the assay; corresponding values for 50 mmol/L ammonium chloride were 15.7% ± 3.0% for washed cells and 46.3% ± 2.7% for cells with base present during stimulation. At these concentrations of bases we did not see functional enhancement, as reported by Phillips et al[15] for iodinating activity of neutrophils washed after treatment with isotonic ammonium chloride.

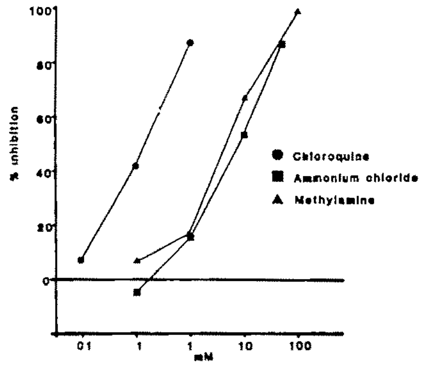

Fig 5. Percent inhibition of f-met-leu-phe-stimulated (0.1 μmol/L) cytochrome c reduction (mean of two to three experiments).

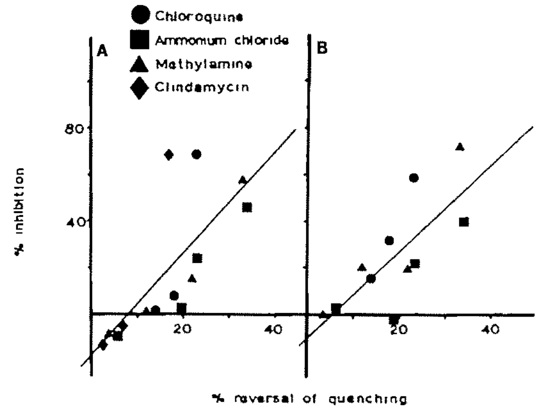

Fig 6. (A) Regression line for dependence of weak base inhibition of cytochrome c reduction upon the degree of lysosomal alkalinization (correlation coefficient $r = .747$, $P < .01$). Stimulus, 5 ng/mL PMA. (B) Regression line for stimulus of 1 μmol/L A23187. $r = .752$, $P < .01$.

Effect of osmolarity and ionic strength. To assess the contribution of hyperosmolarity and increased ionic strength to the weak base effects, sucrose or NaCl was added to the (already isotonic) reaction mixture. The addition of NaCl up to 50 mmol/L and sucrose up to 100 mol/L had little or no effect on superoxide production stimulated by 5 ng/mL PMA. Moderate inhibition was seen when these concentrations were doubled: 33.8% ± 4.6% with 200 mmol/L sucrose and 31.9% ± 19.8% with 100 mmol/L NaCl. Thus, only extreme hyperosmolarity produced inhibition comparable to that seen for lower concentrations of weak bases.

Superoxide scavenging. The xanthine-xanthine oxidase assay was used to detect scavenging of superoxide by weak bases. There was no significant inhibition of cytochrome c reduction in this system by any of the weak bases tested at the highest concentrations used. Indeed, values higher than the control were obtained in some experiments (39.4% ± 24.8% greater than the control in the presence of 1 mmol/L chloroquine and 22.3% ± 15.2% greater than the control in the presence of 50 mmol/L NH_4Cl). To evaluate whether this represented genuinely enhanced superoxide detection in the presence of chloroquine and NH_4Cl, assays were repeated using a different lot of xanthine oxidase (0.53 U/mL) and 10 μmol/L cytochrome c, with OD measurements every 10 seconds to permit calculation of an initial linear rate of cytochrome c reduction. The results showed −3.4% ± 8.0% inhibition by 50 mmol/L NH_4Cl and 8.2% ± 7.8% inhibition by 1 mmol/L chloroquine. Thus, there was no consistent augmentation of cytochrome c reduction, and the data taken together indicate no significant effect on the detection of superoxide in the presence of any of the bases used.

NADPH oxidase activity in particulate fractions. Table 2 shows the effect of ammonium chloride and chloroquine on the NADPH oxidase activity of particles from zymosan-stimulated neutrophils. Both bases produced marked inhibition of activity when added to cells before stimulation, but not when added to intact cells after stimulation or to particle preparations before the assay of NADPH oxidase activity. Relative potency of the two bases was similar to their inhibitory effect on zymosan-stimulated superoxide production by intact cells in suspension.

Kinetic constants for NADPH oxidase, determined using NADPH concentrations from 0.01 to 2 mmol/L, are shown in Table 3. Cells without added base had a mean K_m of 0.09 ± 0.02 mmol/L and a V_{max} of 42.8 ± 13.6 nmol cytochrome c reduced per minute per mgm protein ($n = 7$; no significant difference between controls for bases added before and after stimulation). The addition of bases did not affect the K_m but profoundly reduced the V_{max} if and only if they were added before the cells were stimulated. This suggests that bases did not alter the activity of the NADPH oxidase complex after it was formed and that the presence of bases during cell stimulation led to the production of a reduced quantity of enzyme with normal substrate affinity.

In several experiments, very high concentrations of NADPH (usually more than 20 times the K_m) produced decreased activity and deviation from the Michaelis-Menten kinetics used to derive the kinetic constants. These data were reanalyzed by fitting the equation $V = (V_{max} \times S)/(K_m + S + S^2/K_I)$ for substrate inhibition to determine K_m, V_{max}, and the inhibition constant K_I.[16] The results (Table 4) yielded K_m values comparable to those obtained assuming Michaelis-Menten kinetics at lower substrate concentrations and suggested significant substrate inhibition only at NADPH concentrations far higher than the K_m. The influence of ammonium chloride on V_{max} was unchanged using this procedure.

Table 2. Effect of Bases Added at Various Times on NADPH Oxidase Activity

	Percentage of Control Activity	
Time of Addition	Chloroquine (1 mmol/L)	NH_4Cl (50 mmol/L)
Before activation	17.3 ± 11.6	40.9 ± 1.9
After activation, before disruption	91.1 ± 16.9	115.0 ± 26.1
After disruption	89.0 ± 9.1	94.7 ± 6.1

All values represent the mean ± SEM of two or more experiments. Rates are cytochrome c reduction expressed as a percentage of simultaneous control cells processed identically except for the absence of added base, adjusted for equal protein content, and assayed in the presence of 0.1 mmol/L NADPH

Table 3. The Effect of Weak Bases on Kinetic Constants for NADPH Oxidase

	Control	Chloroquine (1 mmol/L)	NH_4Cl (50 mmol/L)
Bases added before stimulation			
K_m (mmol/L NADPH)	0.07 ± 0.03	0.05 ± 0.01	0.06 ± 0.02
V_{max} (% of control)	100	15.4 ± 11.0	38.8 ± 0.04
Bases added after stimulation			
K_m (mmol/L NADPH)	0.12 ± 0.02	0.12 ± 0.02	0.11 ± 0.03
V_{max} (% of control)	100	87.1 ± 21.4	86.3 ± 9.0

Effect of weak bases on neutrophil oxygen consumption. Oxygen consumption was measured to determine whether the entire sequence of oxidative metabolism was affected by weak bases. The estimated consumption by control cells was 38 ± 14 nmol/5 × 10^6 cells after stimulation by f-met-leu-phe and 22 ± 5 nmol/5 × 10^6 cells after stimulation by PMA. As Table 5 indicates, oxygen consumption was decreased in the presence of bases. As with superoxide production, chloroquine was a more potent inhibitor than ammonium chloride on a molar basis, and less inhibition was seen in PMA-stimulated cells than in f-met-leu-phe–stimulated cells.

Effect on hydrogen peroxide release. Cells treated with ammonium chloride released less hydrogen peroxide after stimulation with f-met-leu-phe (0.1 μmol/L) than control cells, as shown in Fig 7. The effect on the initial rate of release was most striking, again suggesting that the immediate response of the cell to stimulation is affected rather than a subsequent secondary event in oxidative metabolism.

DISCUSSION

When neutrophils are activated by phagocytic stimuli or soluble agents, a respiratory burst is initiated, with resulting production of reactive oxygen metabolites that are toxic to ingested microorganisms. This process involves an increase in oxygen consumption,[17] production of the superoxide anion,[18] and its dismutation to hydrogen peroxide.[13] These metabolites can then react further to produce hydroxyl radicals[18] and active halogenated species such as chlorinating agents[19,20] that are thought to be the principal microbicidal reaction products.

Using superoxide production as an indicator of respiratory burst activity, we have demonstrated that permeant weak

Table 4. Kinetic Constants for Substrate Inhibition of NADPH Oxidase Activity

	K_m (mmol/L NADPH)	K_I (mmol/L NADPH)	V_{max} (percentage of control)
All controls	0.11 ± 0.02	8.6 ± 1.5	100
NH_4Cl			
Before stimulation	0.08 ± 0.02	12.4 ± 2.3	38.7 ± 5.2
After stimulation	0.12 ± 0.04	10.4 ± 3.8	85.2 ± 11.9
Chloroquine after stimulation	0.12 ± 0.02	17.2 ± 3.6	83.1 ± 19.7

Each value represents the mean ± SEM of two to seven experiments. No values were obtained for chloroquine-pretreated cells because the total activity was very low and no definite substrate inhibition was demonstrated.

Table 5. Effect of Bases on Neutrophil Oxygen Consumption

Base	Stimulus	Δ pO$_2$* Control	Base	Inhibition by Base
Chloroquine	PMA	13.8	9.5	29 6%
Chloroquine	f-met-leu-phe	17.2	6.8	62.4%
NH_4Cl	f-met-leu-phe	16.0	11.2	21.7%

*mm Hg decline in recorded pO$_2$ over ten minutes after the addition of stimulus Each value represents the mean of two experiments, each performed in duplicate

bases, which raise the intralysosomal pH in neutrophils[3] as well as other phagocytic cells,[21] inhibit neutrophil oxidative metabolism. This inhibition was dose dependent, was decreased by increasing the concentration of some stimuli, and could be observed using five different stimuli. Washing away the bases largely reversed their inhibitory effect.

Assessment of other components of the respiratory burst showed that oxygen consumption and hydrogen peroxide release were also inhibited. Similar patterns of inhibition were seen when cells were disrupted for analysis of NADPH oxidase activity after activation by a particulate stimulus (opsonized zymosan) and even though oxygen consumption and NADPH oxidase activity were assayed at room temperature after the cells were incubated at 37 °C with weak bases. Kinetic analysis of the NADPH oxidase suggested the formation of reduced amounts of active enzyme having normal substrate affinity.

There are several possible mechanisms for this inhibition. The simplest would be scavenging of superoxide by weak bases, with resulting failure to detect the presence of the superoxide anion by measuring cytochrome c reduction. In such a situation we would be measuring not a true inhibition of cellular function but an impairment of our ability to detect the products of stimulated cells. The xanthine-xanthine oxidase assay was used to test this possibility. Since none of the weak bases produced any impairment of superoxide detection in the cell-free system, scavenging of the superoxide anion cannot explain our results. The impairment of oxygen consumption and H_2O_2 production also argues against this possibility, as does the fact that particles from amine-loaded cells had diminished NADPH oxidase activity although most of the base was washed away during preparation of the particles.

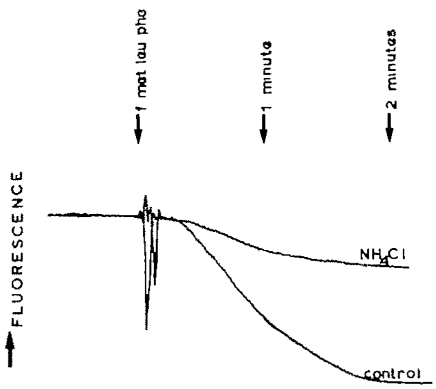

Fig 7. Effect of 60 mmol/L NH_4CL on H_2O_2 production (indicated by reduction in scopoletin fluorescence) after stimulation of neutrophils by f-met-leu-phe. The artifact immediately after the addition of f-met-leu-phe is due to the insertion of the pipette tip into the cuvette for mixing. Tracing is representative of three experiments in which the lag time was 21 ± 4 seconds for the control and 24 ± 2 seconds for NH_4Cl-treated cells. The initial rate was 1.21 ± 0.08 nmol/min/10^6 control cells with 55.6% ± 22.0% inhibition by NH_4Cl, and the total H_2O_2 production for a period of five minutes was 2.31 ± 0.45 nmol/10^6 cells with 40.0% ± 26.7% inhibition by NH_4Cl.

Another explanation on the basis of artifact would be that very large amounts of superoxide are produced and dismuted to hydrogen peroxide, which in turn reoxidizes the reduced cytochrome c. This could not explain the reduction of oxygen uptake and hydrogen peroxide release. Thus, our findings appear to reflect a genuine depression of cellular activity.

A second hypothesis would be that the addition of weak bases to a balanced salt solution produces a rise in osmolarity and/or ionic strength sufficient to inhibit neutrophil function. Although we were able to inhibit superoxide production with extremely high concentrations of sucrose or NaCl, we saw remarkably little inhibition using up to 50 mmol/L NaCl or 100 mmol/L sucrose. It is unlikely, therefore, that these factors make a major contribution to the inhibition observed using weak bases, although they may play a role at the highest methylamine concentrations studied.

A third possibility is that weak bases interfere with binding of stimuli to membrane receptors. In this case inhibition might be expected to be stimulus specific. We observed inhibition of superoxide production in response to four different stimuli that are presumed to activate the respiratory burst by different mechanisms.[22-25] In particular, inhibition was seen with an ionophore stimulus (A23187) and a halide stimulus (fluoride anion) as well as with receptor-mediated activation (zymosan, f-met-leu-phe). The fact that the phenomenon is so general argues against the likelihood that inhibition results purely from interference with stimulus-receptor binding.

A fourth possibility is suggested by recent work of Thomas and co-workers.[26] These authors propose that when the neutrophil respiratory burst is activated in the presence of permeant amines, hypochlorous acid produced as a result of the respiratory burst reacts with the amines to form toxic nitrogen-chlorine derivatives. These compounds then diffuse into the cell where they produce oxidative damage to cell components and inhibition of further cellular activity. Clark and Borregaard[27] and Ohno and Gallin[28] have proposed that direct inactivation of neutrophil products by oxygen metabolites and similar processes might likewise be augmented by the presence of amines. Such a sequence of events could explain many of our results, although we have not seen increased cell death when neutrophils are activated in the presence of permeant amines[3] (also unpublished observations, 1983) and the reversal of inhibition by high concentrations of PMA would be somewhat surprising in this context. If this were the explanation, we would also expect to see inhibition of NADPH oxidase activity in particles from cells treated with bases after stimulation, but we observed no such effect. An additional argument against this hypothesis is the immediate inhibition of hydrogen peroxide release we observed, which would not be expected if cell damage occurs only after the backdiffusion of the initial products of oxidative metabolism.

We suggest that the inhibition of oxidative metabolism, which correlates with lysosomal alkalinization by weak bases, is at least in part a direct result of that alkalinization. This hypothesis is in accordance with the significant correlation between release of 9-aminoacridine quenching and inhibition of superoxide production. It also explains the substantial reversal of inhibition when alkalinization is reversed by washing away the base.

By what mechanism does a disturbance of lysosomal pH regulation lead to suppression of the respiratory burst? One possibility is that these results reflect a general alteration of membrane fusion processes. The existence of an acid compartment and transmembrane pH gradient has been shown to be either a prerequisite or a concomitant factor in many processes involving fusion. For example, the Semliki Forest virus can be induced to fuse with cell membranes by low pH treatment.[1] Low pH promotes fusion of small unilamellar vesicles in synthetic lipid-membrane systems.[29] Rapid acidification of endocytic vesicles takes place not only in the neutrophil phagolysosome[30-32] but also in mouse fibroblasts[33] and single-cell organisms.[34,35] Permeant weak bases such as methylamine and ammonium chloride, which should alkalinize the lysosome[36] and/or the intermediary endocytic vesicle, have been reported to inhibit phagosome-lysosome fusion[37] and to block various steps in receptor-mediated endocytosis including receptor clustering into coated pits[38] and delivery of internalized ligand to the lysosome.[39] Another method of alkalinizing acidic intracellular vesicles using the ionophore monensin has likewise been reported to block delivery of endosome contents to the macrophage lysosome.[40] In addition to phagosome-lysosome fusion, our previous results[3] suggest that lysosomotropic weak bases inhibit lysosome-plasma membrane fusion and thus impair neutrophil degranulation. Although others have reported more variable effects of weak bases on neutrophil function, the relationship between pH change and functional effects was not explicitly examined.[41]

Reduction of the pH gradient across the lysosomal membrane may therefore explain inhibition of neutrophil oxidative metabolism by weak bases to the extent that the respiratory burst depends upon lysosome/plasma membrane fusion. Activation of the enzyme complex responsible for production of reactive oxygen metabolites may involve assembly of a variety of components: those that have been proposed include a flavoprotein, a b-type cytochrome, ubiquinone, and an unidentified chromophore.[8,42-46] The assembly process may involve the fusion of plasma membrane with subcellular membranes and the transfer of components from one membrane location to another. For example, flavin adenine dinucleotide reportedly is present in plasma membrane and specific granules of resting cells but is increased in phagolysosomes relative to the plasma membrane.[42] Transfer of ubiquinone from granules to phagolysosome has been proposed as an important step in the respiratory burst.[43] There is evidence that activation of the NADPH oxidase involved in neutrophil superoxide generation may require translocation of cytochrome b from granule membranes to the plasma membrane,[47] including a report that abnormal localization of cytochrome b in the plasma membrane may be associated with a supranormal respiratory burst.[48] Thus, by impairing membrane fusion, lysosomal alkalinization could interfere with assembly of the active enzyme complex. This is not an all-or-none effect, as demonstrated by the failure to abolish superoxide production completely and the lessening of inhibition at high-stimulus concentrations. This in turn would be consistent with the report that granule-depleted cytoplasts are capable of oxidative metabolism,[49] which implies that the requirement for granule-plasma membrane interaction may not be absolute. That is, the elements necessary for enzyme assembly may all be present in plasma membrane, as suggested by analysis of cytoplasts,[50] but augmented by further supplies from granule fusion. A recent report that PMA produces less cytochrome b translocation relative to superoxide release than either A23187 or f-met-leu-phe/cytochalasin B[51] would then be compatible with our finding that cells stimulated by high concentrations of PMA show relatively less inhibition by bases than cells activated by other stimuli. The ability to overcome inhibition at very high PMA concentrations is consistent also with the reported graded dose-response of oxidative metabolism to PMA[52] and may reflect recruitment of additional receptor sites or of alternative pathways of enzyme activation. Failure to reverse the chloroquine effect completely at high-PMA concentrations suggests that this particular base may have direct effects on receptor interactions in addition to the pH-dependent effects that it shares with other bases, and other studies have been undertaken to elucidate this.

Measurement of NADPH oxidase activity in particulate preparations from stimulated neutrophils did suggest that lysosomotropic weak bases interfere with the assembly of the active enzyme complex, but without altering its substrate affinity. We speculate that this may reflect alterations in the intracellular calcium flux that occurs during neutrophil activation by diverse stimuli. In support of this hypothesis, recent data from this laboratory[53,54] indicate that alkalinizing weak bases can modify the rise in cytosolic calcium associated with activation. Studies are in progress to delineate the step in the activation sequence at which weak base inhibition of neutrophil function occurs and to further elucidate the mechanism of inhibition.

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Receptor Patching and Capping of Platelet Membranes Induced by Monoclonal Antibodies

By S. Santoso, U. Zimmermann, J. Neppert, and C. Mueller-Eckhardt

Redistribution of glycoproteins (GP) Ib, glycocalicin, IIb, and IIIa on the surface of human platelets in response to stimulation with corresponding monoclonal antibodies (MoAb) and a polyclonal antiglycocalicin antibody was studied by immunofluorescence, immunoelectron microscopy, and a quantitative radioimmune assay. Immobilization of the antigens by prefixation with formaldehyde showed a uniform distribution over the surface of the platelet. Incubation of unfixed platelets with specific MoAb against various epitopes on GPIIb and/or IIIa resulted in a time-dependent patching, subsequent capping, and after prolonged exposure to the antibody/label complex, internalization of the complex, possibly by endocytosis. In contrast, GPIb could not be shown to cap. From these results we conclude that platelet GPIIb and/or IIIa undergo spatial rearrangement in a manner analogous to that observed in lymphocytes, whereas GPIb does not. Since both GPIb and GPIIb and/or IIIa seem to be transmembraneous GP associated with the cytoskeleton, a special, though unidentified, role of GPIIb/IIIa in the induction of lateral membrane mobility is postulated.

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PLATELETS participate in a variety of immunologic reactions. They not only serve as immunologic targets because of their antigenic membrane constituents but also contribute to the inflammatory response accompanying tissue injury. Platelets accumulate in vessels adjacent to inflammatory sites and interact with bacteria, viruses, and antigen-antibody complexes.[1] These functions are mediated by different membrane receptors, the majority of which are assumed to be glycoproteins (GP).[2]

From an immunologic point of view, two major GP are of particular importance. GPIb appears to be associated with the platelet Fc receptor[3,4] and with a receptor for quinine/quinidine–dependent platelet-specific antibodies.[5] At the same time, GPIb (together with GPV) is believed to represent a thrombin receptor.[2] GPIIb/IIIa, a macromolecular complex involved in platelet aggregation, is the site of the platelet-specific antigens $\mathrm{Pl}^{A1/A2}$ (or $\mathrm{Zw}^{a/b}$).[6] More recently, both GPIb and IIb/IIIa have been reported to carry autoantigens against which platelet-specific autoantibodies may be directed.[7,8]

Recent studies have shown that upon stimulation by thrombin[9] or by concanavalin A (Con A)[10] platelet surface membrane receptors undergo rearrangement by patching and capping in a manner analogous to that described in the lymphocyte system. However, it was not yet known whether specific antibodies can exert the same effect and which of the major GP of the platelet membrane are implicated in these reactions. The availability of monoclonal antibodies (MoAb) directed toward specific platelet epitopes[11,12] prompted us to study the distribution, mobility, and possible redistribution of platelet GPIb and GPIIb/IIIa. These GP were chosen because of their presumed participation in platelet immune reactions. Using immunofluorescence, immunoelectron microscopy (IEM) techniques, and a quantitative radioimmune assay, we show here that GPIIb/IIIa undergo a reorganization and subsequent internalization upon stimulation with MoAb, whereas GPIb does not.

MATERIALS AND METHODS

Platelets. Platelet-rich plasma (PRP) from the citrated blood of healthy donors was used. Platelets were washed using a Tyrode buffer system (TB [Merck, Darmstadt, FRG]) containing Ca^{2+} and prostaglandin (PGE1 [Serva, Heidelberg, FRG]) as described previously.[13]

Antibodies. Designation, origin, and specificity of MoAb against GP of human platelets are given in Table 1. MoAb were purified from ascites by protein A–sepharose 4B chromatography before use. MoAb were used at a concentration of 1 μg/mL and were free from aggregates as demonstrated by enzyme-linked immunosorbent assay and cross-immunoelectrophoresis (data not shown). Rabbit antiglycocalicin was kindly supplied by Dr Clemetson, Bern, Switzerland. Goat antimouse (GAM) IgG $F(ab')_2$ fragments conjugated with fluorescein isothiocyanate (FITC) or tetrarhodamine isothiocyanate (RITC), both from Cappel Laboratories, West Chester, Pa, and FITC goat antirabbit IgG (Behringwerke, Marburg, FRG) served as secondary antibodies. These conjugates were used at a dilution of 1:50. An affinity-purified rabbit antimouse (RAM) IgG antibody and protein A–gold particles (diameter, 6 nm) were generously supplied by Dr G. Griffiths, European Molecular Biology Laboratory, Heidelberg, FRG. Affinity purified GAM IgG or rabbit antigoat (RAG) IgG $F(ab')^2$ fragments, both from Dianova, Hamburg, FRG, served as secondary or tertiary antibodies, respectively, and were labeled with Na ^{125}I using the iodine monochloride method.[18] Generally, 1 mg protein was labeled with 1 mCi Na ^{125}I. The ratio of protein-bound ^{125}I to free ^{125}I was 98%.

Immunofluorescence microscopy. For labeling of GPIb or GPIIb/IIIa, 500 μL unfixed washed platelets ($3 \times 10^5/\mu L$) were incubated with 50 μL MoAb or rabbit antiglycocalicin IgG, respectively, for ten or 30 minutes at 37 °C and were then fixed with 1% formaldehyde (freshly prepared from paraformaldehyde) in TB. To demonstrate the distribution of the antigens on resting platelets, samples were first fixed, rinsed with TB, and then incubated with MoAb or rabbit antiglycocalicin for 30 minutes at 37 °C. Thereafter, all samples were rinsed and incubated with 30 μL RITC-GAM for 30 minutes at 20 °C and finally washed twice with TB. Receptor internalization was studied by incubating unfixed platelets with MoAb for 30 minutes. Samples were then rinsed, incubated with RITC-GAM for ten minutes at 20 °C, fixed, and finally washed

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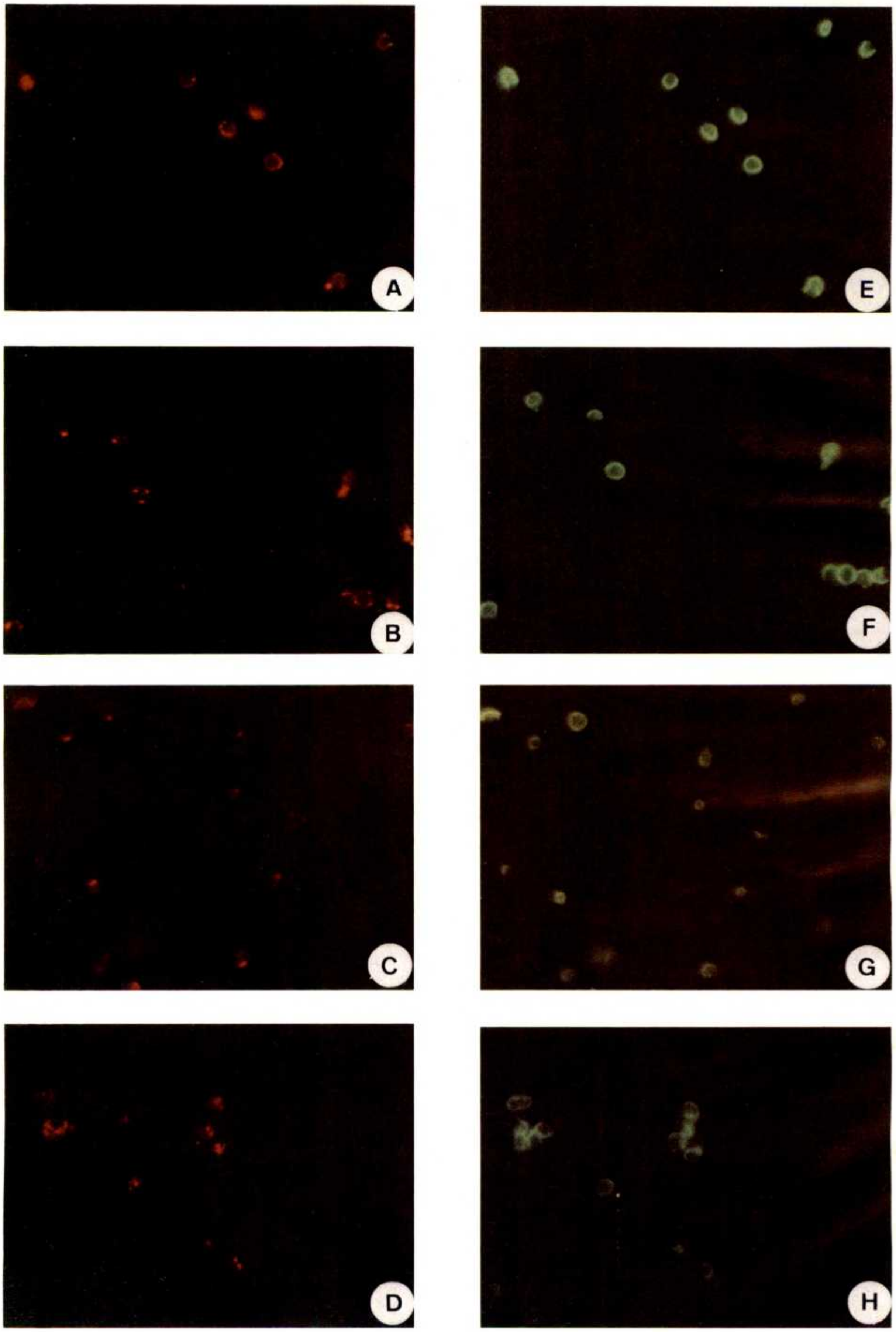
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Table 1. Antibodies Used for Labeling of GPIb or GPIIb/IIIa on Human Platelets

Designation of Antibody	Immunoglobulin	Specific for Various Epitopes on	Reference
MoAb Gi5	mouse IgG2a	GPIIb/IIIa	Santoso et al[11]
MoAb 2G12	mouse IgG2a	GPIIb/IIIa	Woods et al[7]
MoAb 3F5	mouse IgG1	GPIIb/IIIa	Woods et al[7]
MoAb C15	mouse IgG1	GPIIIa	Tetteroo et al[14]
MoAb C17	mouse IgG1	GPIIIa	Tetteroo et al[14]
MoAb FMC 25	mouse IgG1	GPIb, IX	Zola et al[15]
MoAb AN51	mouse IgG	GPIb	McMichael et al[16]
Polyclonal	rabbit IgG	glycocalicin	Ali-Briggs et al[17]

twice with TB. Normal mouse IgG (Cappel Laboratories) served as a negative control.

For differential staining, GPIb and GPIIb/IIIa were investigated by double immunofluorescent labeling: platelets treated with MoAb against GPIIb/IIIa, according to the preceding procedure, were incubated with 50 μL MoAb against GPIb for ten minutes at 20 °C and washed twice in TB. Finally, the platelets were labeled with 30 μL FITC-GAM for 30 minutes at 20 °C. Labeled samples in TB/glycerin were examined and photographed with a Zeiss Universal microscope equipped with an epi-illuminator, FITC-, RITC-selective filters, and a 40 × Plan-Neofluar and 63 × Plan-Apochromat objectives. Micrographs were made on Kodak Ektachrome 400 (commercially processed for 33 DIN [Deutsche Industrie Norm]) at final magnifications of 400 to 600×.

IEM. Fixed or unfixed platelet samples that had been incubated with antibodies, as previously mentioned, were labeled with protein A–colloidal gold for 20 minutes at 20 °C. Prior to gold labeling, MoAb-treated samples were incubated with RAM for 30 minutes at 37 °C and rinsed in TB. This step was used to facilitate the binding of protein A. Samples were rinsed again, fixed with 2% OsO_4 for 30 minutes at 4 °C, and dehydrated rapidly in acetone. Contrast was enhanced by treatment with 2% uranyl acetate for two minutes in 50% acetone, and samples were embedded in Epon. Serial thin sections were treated with 2% uranyl acetate and 2% lead citrate to further enhance contrast and were observed and photographed with a Zeiss EM 10 CR electron microscope.

Radioimmunoassay. To demonstrate endocytosis quantitatively, 5×10^7 unfixed washed platelets were incubated with 50 μL MoAb for ten minutes at 20 °C. After washing with TB, the samples were incubated with 50 μL of either radiolabeled (20,000 cpm; series A) or unlabeled GAM (dilution, 1:1,000; series B) for 5, 10, 30, 60, and 90 minutes at 20 °C, were then fixed, and washed three times with TB. Series A samples were transferred to counting tubes, and the bound radioactivity was determined in a gamma counter (Abbot Laboratories, Eschborn, FRG) These values represented the total amount of GPIb or IIb/IIIa, respectively, detectable at the outer surface of the platelets as well as after internalization. To discriminate between these two compartments, series B samples were tagged with 50 μL of ^{125}I-RAG (20,000 cpm) for ten minutes at 20 °C and were further assessed as previously described. These values equaled the amount of GP that had remained at the external surface prior to fixation. The difference of radioactivity between corresponding samples of series A and B was taken as a measure of internalization of the respective GP. All tests were run in duplicate.

RESULTS

Surface distribution of GPIb and GPIIb/IIIa on fixed platelets. Immobilization of antigens by formaldehyde fixation before incubation with MoAb Gi5 allows the visualization of GPIIb/IIIa distribution in the resting state. As seen in Fig 1A, the fluorescence appeared rather diffuse and uniform over the platelet surface with only minute irregularities. The same distribution pattern was seen in double fluorescence (Fig IE) using MoAb FMC 25 directed against GPIb. Colloidal gold labeling of GPIIb/IIIa in IEM (Fig 2A) also demonstrated a more or less uniform distribution of this antigen on the plasma membrane. The slight inhomogeneity in the distribution of GPIIb/IIIa on fixed platelets (Figs 1A and 2A) is probably a sequel of multiple platelet washing.

Distribution of GPIb and GPIIb/IIIa after MoAb stimulation. Incubation of unfixed platelets with MoAb Gi5 for ten minutes resulted in clustering (patching) of the antigen as seen in fluorescence (Fig 1B) and in IEM (Fig 2B). If, after patching of GPIIb/IIIa, the platelets were counterstained with MoAb FMC 25 and FITC-GAM, GPIb remained uniformly distributed and did not coaggregate with the former antigen (Fig 1F). After 30 minutes' incubation with MoAb Gi5, the majority of labeled platelets were capped, both in fluorescence (Fig 1C) and in IEM (Fig 2C), whereas the distribution of GPIb remained unchanged (Fig 1G). After 40 minutes' incubation time, the antigen-antibody label complex was internalized, presumably by endocytosis. Both fluorescence (Fig 1D) and IEM (Fig 2D) demonstrated intracellular label and loss of the complex from the surface. In contrast, no change became visible in the fluorescence pattern of GPIb (Fig 1H). Control experiments with unfixed platelets incubated with MoAb FMC 25 for ten or 30 minutes did not reveal any capping (data not shown). No patching or capping was found by colloidal gold labeling of GPIb in IEM (Fig 3).

Identical results, not depicted in the Figs, were obtained by substituting 2G12, 3F5, C15, and C17 for Gi5 or AN51 and rabbit antiglycocalicin IgG for FMC 25 (see Table 1).

Internalization of GPII/IIIa (endocytosis). Endocytosis was investigated by a quantitative radioimmune assay. The results of a representative experiment are shown in Fig 4. If unfixed metabolically active platelets were incubated either with MoAb Gi5 (open triangles) or with FMC 25 (open circles), respectively, and then with a radiolabeled secondary antibody (GAM), there was a moderate increase of radioactivity over the five minutes' value (100%) with both MoAb that reached a plateau after approximately 30 minutes. Similar curves were obtained if fixed instead of unfixed platelets were used, indicating that the increase of radioactivity was due to binding kinetics of antibodies and not to a platelet-dependent process. The data also show that the total amount of GP-MoAb-GAM complexes remained constant

Fig 1. Immunofluorescence of fixed (0 minutes) and unfixed platelets labeled 10, 30, and 40 minutes with MoAb Gi5, then with RITC-GAM, followed by MoAb FMC 25 and FITC-GAM. The distribution of GPIIb/IIIa is shown after 0 minutes, even rings (A); ten minutes, patches (B); 30 minutes, caps (C); and 40 minutes, internalization (D). The distribution of GPIb after respective incubation times did not change (E, F, G, H: even rings).

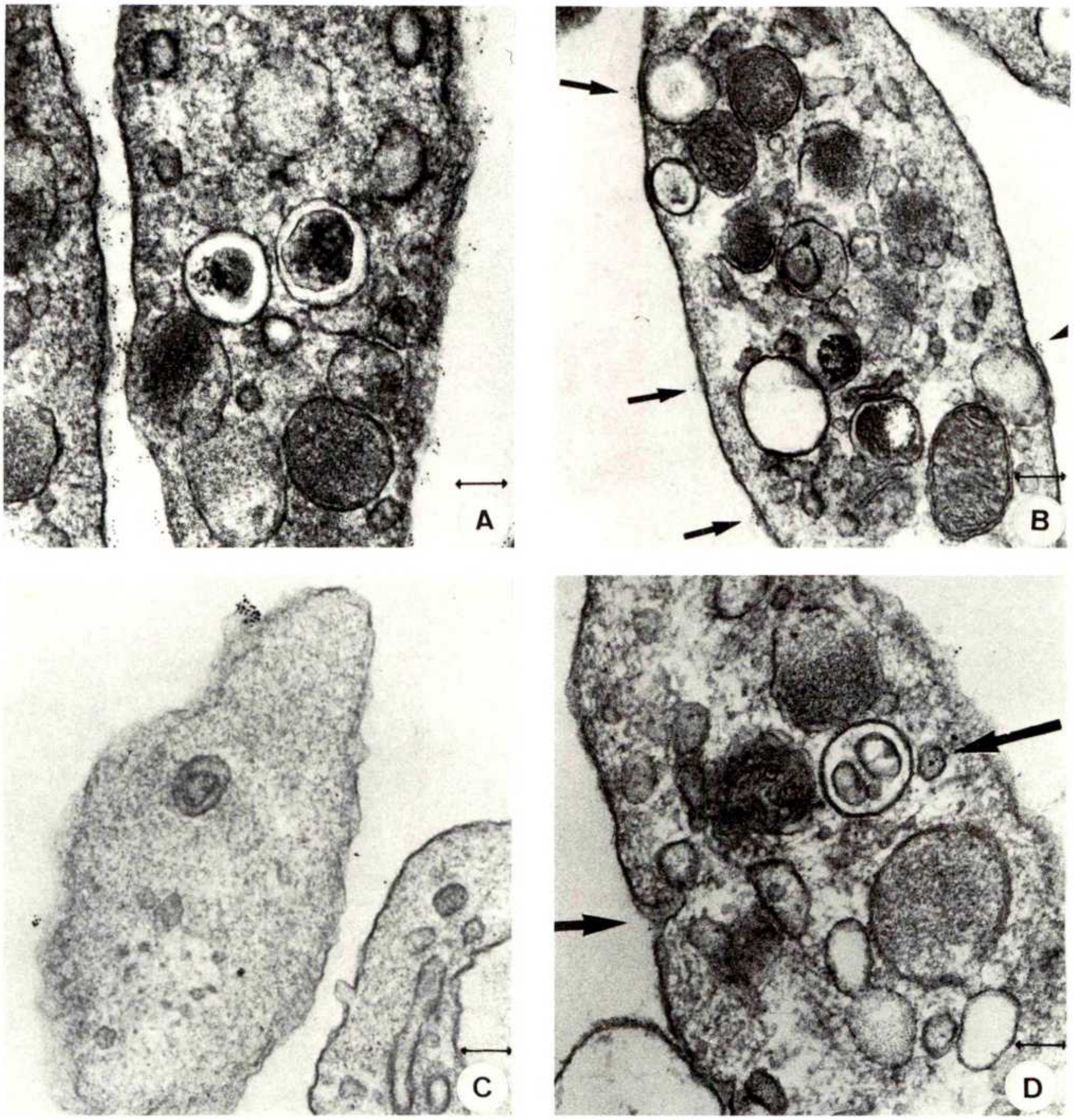

Fig 2. IEM of fixed (0 minutes) and unfixed platelets labeled 10, 30, and 40 minutes with MoAb Gi5, followed by RAM and protein A gold particles. The distribution of GPIIb/IIIa is shown after 0 minutes, randomly scattered (A); ten minutes, gold particles in patches on the cell surface (arrows) (B); 30 minutes, clustered gold particles as caps on the cell surface (C); 40 minutes, invagination of the labeled membranes and formation of vesicles with labeled complexes (arrows) (D). The marker ↔ indicates 1 μm.

during incubation, precluding the possibility of their release into the medium (shedding). The results were strikingly different if unfixed platelets were treated with unlabeled instead of radiolabeled secondary antibody (GAM; closed symbols) prior to fixation and exposure to tertiary radiolabeled antibody (RAG) under otherwise identical experimental conditions (for details see Materials and Methods). In these circumstances the radiolabeled tertiary antibody can only bind to those GAM molecules that are still accessible at the platelet surface. It is evident that there was a rapid, precipitous decrease of radioactivity after pretreatment of platelets with the GPIIb/IIIa-specific MoAb (Gi5), whereas no such change was discernible after pretreatment with the GPIb-specific MoAb (FMC 25). It therefore follows that a large portion of the GPIIb/IIIa-GAM complexes, but not of the GPIb-GAM complexes, had been transferred from the

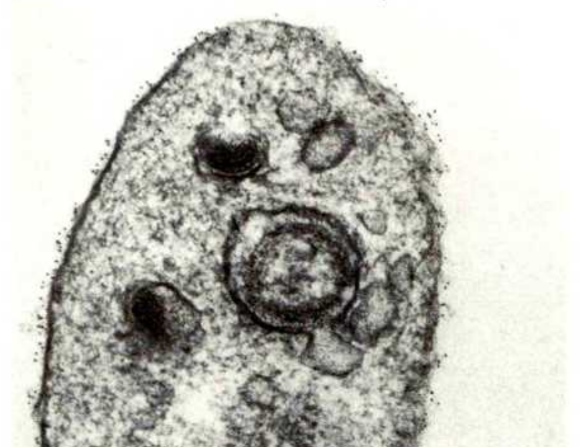

Fig 3. **IEM of unfixed platelets labeled 30 minutes with MoAb FMC 25, followed by RAM and protein A gold particles. The distribution of GPIb remained randomly scattered. The marker ↔ indicates 1 μm.**

platelet surface to the interior of the platelets, which is strong additional evidence for platelet endocytosis.

DISCUSSION

In lymphocytes as well as in other cells it has repeatedly been demonstrated that the redistribution of cell suface macromolecules within the plane of the plasma membrane is caused by receptor-assembly aggregation in response to multivalent ligands. Lectins as well as specific antibodies have been shown to induce patching, a local clustering of receptors, followed by capping, an aggregation of the clusters to a single area on the cell surface.[19] Whereas the former is a passive phenomenon, the latter is an active, energy-dependent process that requires an intimate association of the receptor to the intact cytoskeletal system.[20] Following capping, the molecules can be seen to be internalized by endocytosis.

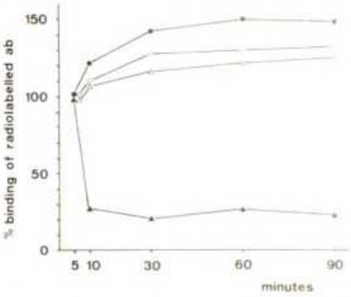

Fig 4. **Demonstration of internalization of GPIIb/IIIa (but not of GPIb) by a quantitative radioimmune assay. Washed platelets were first incubated with MoAb (ten minutes, 20 °C), then with a radiolabeled (open symbols) or an unlabeled secondary antibody (GAM) for various periods of time, and were then fixed. Finally, unlabeled, fixed platelets were treated with a radiolabeled tertiary antibody (RAG; closed symbols). For details see Materials and Methods. Open symbols indicate the total amount of GPIb (○) or IIb/IIIa (△) present either on the surface and/or internalized; closed symbols indicate GP exclusively present on the platelet surface (GPIb, ●; GPIIb/IIIa, ▲). The differences between corresponding values of curves are a measure of GP internalization. It is evident that GPIb remained on the platelet surface while a large proportion of GPIIb/IIIa was internalized.**

Our results show that similar events take place on the platelet membrane in response to MoAb directed against an epitope on GPIIb/IIIa, but not if the MoAb are directed against GPIb. Membrane reorganization occurred in a time-dependent fashion. After approximately 30 minutes, GPIIb/IIIa was largely capped at one polar site of the platelet, and after 40 minutes, was removed from the membrane by apparent internalization (Figs 1D and 1H). In double fluorescence labeling experiments it could be shown that, during capping and endocytosis of GPIIb/IIIa, GPIb remained evenly distributed on the platelet surface. This was further substantiated by a quantitative radioimmune assay using radiolabeled secondary and tertiary antibodies (Fig 4). In controls there was some minor inhomogeneity in GPIIb/IIIa distribution already on fixed platelets. However, the inhomogeneity did not progress upon prolonged incubation before fixation and most likely was due to mechanical stimulation of platelets by multiple washing.

Zucker and Nachmias[21] have recently pointed out that it is basically difficult to prepare platelets in the absolute resting state, and Hourdille et al[22] reported similar clustering phenomena with unactivated platelets.

The distinct behavior of GPIb as opposed to GPIIb/IIIa is difficult to interpret with regard to ligand-dependent reorganization on the platelet surface. From lymphocyte studies it is known that only molecules connected to the cytoskeleton can undergo membrane reorganization. By analogy one would have to assume that the same holds true for platelets. Since there is evidence that both GPIb and GPIIb/IIIa represent transmembrane GP associated to the cytoskeleton, our results lend support for a special, as yet obscure, role of GPIIb/IIIa in the capping process.

Until now there is only sparse information as to the exact location of these GP within the platelet plasma membrane and their association to internal mechanical structures. It has been inferred from biochemical data that GPIIb/IIIa are integral, transmembrane proteins.[2] More recent results by Painter et al[23] have indicated that two forms of GPIIb and III exist, one of which binds directly to endogenous membrane actin and one that does not. It is conceivable that it is the actin-binding portion of the GPIIb/IIIa complex that transmits the glycoprotein-cytoskeleton interactions induced by Con A in the intact platelet.[24] Since a similar effect can be elicited by MoAb against various epitopes on the GPIIb/IIIa complex, as shown here, it is likely that cross-linking of this particular GP constitutes the specific signal for lateral membrane mobility ultimately leading to capping and endocytosis. It furthermore follows that neither cross-linking of membraneous GP as such nor their association with the cytoskeleton alone are sufficient prerequisites for cap induction. Such a concept is corroborated by our experiments using MoAb against GPIb and polyclonal antibodies against glycocalicin. Contrary to previous views, Solum and Olsen[25] have recently provided evidence that GPIb traverses the plasma membrane whereas glycocalicin represents the terminal part of the GP at the outer surface. Okita et al[26] have demonstrated that GPIb is tightly associated with actin-binding protein in an apparent transmembrane complex. Thus, if ligand-induced cross-linking of a transmembrane GP would suffice for cap formation, MoAb against GPIb should have produced the same phenomenon. This was not the case, however. Our data also suggest an indirect effect of thrombin in inducing cap formation[9] since the MoAb FMC 25 directed against GPIb, which is assumed to carry the thrombin receptor, did not elicit cap formation. Our observation that patching, capping, and endocytosis can be triggered in platelets by GPIIb/IIIa-specific MoAb is noteworthy in another respect. Berke and Fishelson[27] have suggested that in mouse L cells and leukemia EL 4 cells capping by Con A or antibodies could easily be induced in intact cells, but virtually not in nucleus-free cytoplasts enucleated with the aid of cytochalasin B. They concluded that capping of cell surface receptors depends on their interaction with a nucleus-associated structure. Since human platelets are released from the cytoplasm of megakaryocytes of the bone marrow and do not possess nuclei, our results clearly oppose such a hypothesis and favor the concept that cap formation is a nucleus-independent membrane process.

The findings presented here may provide a plausible explanation for some as yet poorly understood phenomena. If cap formation is regarded as a membrane-clearing process, one could hypothesize that platelet antibodies often directed against epitopes on GPIIIa[6,7,28] may stimulate capping and subsequently be actively removed from the membrane in vivo by endocytosis, which in turn could assist the platelet to escape sequestration in the reticuloendothelial system. Such an assumption might explain why the concentration of platelet-associated IgG is normal in about 30% of patients with chronic autoimmune thrombocytopenia.[29,30] On the other hand, circulating immune complexes bound to the Fc receptor on GPIb may be nonstimulatory for cap induction and therefore can remain detectable at high concentrations on circulating platelets. High levels of platelet-associated IgG is a frequent finding in patients with systemic lupus erythematosus, considered to be an immune complex disease, as well as in patients with sepsis or infectious conditions.[31,32] Naturally, this hypothesis can only remain valid if GPIb does in fact contain the Fc receptor, which at this point is not certain.[33]

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Somatic Shift to Homozygosity for a Chromosomal Polymorphism in a Child With Acute Lymphoblastic Leukemia

By Judith Stamberg, Ashok Shende, and Philip Lanzkowsky

A 12-year-old girl with acute lymphoblastic leukemia (ALL) had two types of acquired cytogenetic abnormalities in her pretreatment peripheral blood and bone marrow: hyperdiploidy due to tetrasomy 8, 10, and 21; and, in the hyperdiploid cells, a shift from heterozygosity to homozygosity for a polymorphic variant on chromosome 15. Both abnormalities disappeared after chemotherapy, when the patient entered clinical remission. It has recently been found that shifts to homozygosity occur in retinoblastoma and Wilms' tumor. Our observation extends this finding to leukemia and indicates that such shifts may have general importance in tumorigenesis.

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IT HAS BECOME clear in recent years that malignant clones often exhibit chromosome abnormalities, and that these acquired abnormalities are often highly specific for a particular type of tumor. Translocations, as well as deletions of entire chromosomes or of specific regions, are well-known findings in chronic myelogenous leukemia, meningioma, and retinoblastoma, for example. Disturbances of chromosome segregation leading to loss or gain of apparently random chromosomes, and consequently to hypodiploidy or hyperdiploidy, are frequently found in acute myeloblastic leukemia (AML) and acute lymphoblastic leukemia (ALL). Other chromosomal mechanisms, leading to genetic homozygosity, have very recently been shown to be operating in retinoblastomas and Wilms' tumors. The use of cloned DNA probes homologous to regions of chromosome 13q and chromosome 11p, respectively, has shown that regions that are heterozygous in unaffected cells of individuals with tumors are homozygous in the tumor cells themselves. Mitotic nondisjunction and duplication (leading to loss of an entire chromosome, duplication of its homolog, and consequent homozygosity for the entire chromosome) as well as mitotic recombination (leading to homozygosity for only that area of the chromosome distal to the recombination event) may both play a role in this shift from genetic heterozygosity to homozygosity.

We report here on a young girl with ALL who had two types of chromosome abnormalities in her malignant clone: hyperdiploidy due to tetrasomy 8, 10, and 21; and a shift from heterozygosity to homozygosity for a large chromosome polymorphism on chromosome 15p. To our knowledge this type of cytogenetic event has not been previously reported.

MATERIALS AND METHODS

Case Report. A 12-year-old white girl was seen for complaints of fever, lethargy, anorexia, abdominal pain, and pallor. Her past history and family history were unremarkable. On physical examination, she was found to have hepatosplenomegaly and petechiae on her palate. A complete blood count showed a hemoglobin of 9.1 g/dL; hematocrit of 26%; platelet count of 12,000/μL; WBC count of 55,700/μL with differential count of leukemic blast cells 88%; promyelocytes 2%; stabs 1%; segmented neutrophils 2%; and lymphocytes 7%. Most of the leukemic blasts had predominantly cytoplasmic vacuolation; however, some degree of nuclear vacuolation was observed in some of the cells. Cytoplasm was abundant with light-blue to dark-blue staining with Wright's stain. Most of the blast cells were devoid of cytoplasmic granules; however, a minority of the blast cells (25% of the total number of blast cells) contained cytoplasmic granules. No Auer rods were seen. Nuclear chromatin was immature and the nuclei contained one to three prominent nucleoli.

Cytochemical staining of leukemic blasts showed: myeloperoxidase-negative; periodic-acid Schiff (PAS)-positive in 79% of blast cells and negative in 21% of blast cells; Sudan black-positive in 11% of blast cells and negative in 89% of blast cells; chloracetate esterase-negative; nonspecific esterase-negative; and acid phosphatase-negative. Immunological surface marker analysis of blast cells from bone marrow and blood showed HLA-DR (Ia) antigen and B4 antigen positivity and other lymphoid lineage markers (common ALL antigen, B1, B2, SIgM, Leu-1, Leu-4, T_{11} and AET rosettes) negativity. Myeloid lineage antigens (MO_1 and MO_2) were also negative. Terminal deoxynucleotidyl transferase was present in only 50% of blast cells.

Although blast cells of this patient were observed to contain both PAS-positive and Sudan black-positive populations, the diagnosis of AML was excluded on the basis of negative myeloperoxidase and negative specific and nonspecific esterase staining. Thus, the final diagnosis of ALL with positivity for HLA-DR (Ia) and B_4 antigens and negativity for common ALL antigen (CALLA) and other lymphoid lineage antigens was made. The patient was placed on a modified Berlin-Frankfurt-Muenster protocol,[1] which uses vincristine, daunomycin, L-asparaginase, prednisone, and intrathecal ara C and methotrexate during induction phase. She began treatment four days after her initial evaluation and completed the induction phase of treatment 27 days later. She attained remission successfully and continues to be in complete remission twelve months later.

Cytogenetic Studies. Samples of blood or bone marrow were received on five occasions during the six-week period from the patient's first referral until after remission was achieved. In accordance with institutional guidelines, informed consent for this and other standard diagnostic procedures was obtained at the time of referral. Blood samples were split into parallel cultures, with and without mitogen stimulation, and were grown for 72 hours. However, the unstimulated preparations yielded no mitotic cells. Bone marrow was studied in direct preparations without mitogen stimulation. Standard cytogenetic techniques[2] were used, followed by trypsin-Giemsa banding.

Samples 1 and 2 were peripheral blood and bone marrow, respectively, received prior to the start of therapy. Two populations were found in both tissues. Sixteen of 20 cells (80%) in blood, and 19 of 27 cells (70%) in marrow, had a normal female karyotype. A very large, prominent p arm on one No. 15 chromosome was noted in

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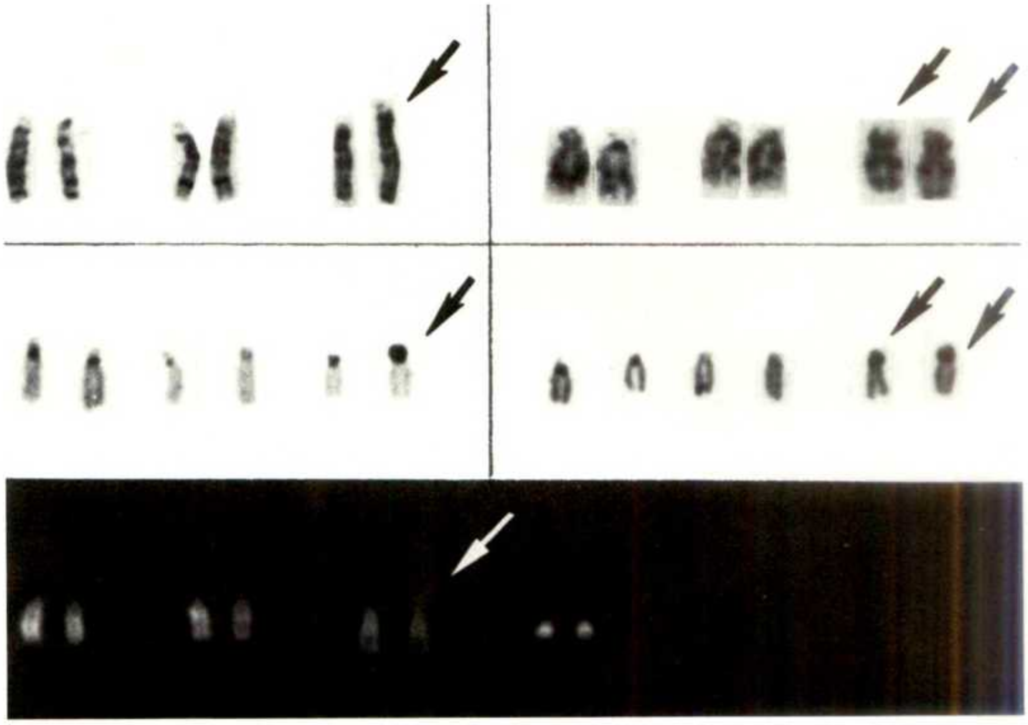

Fig 1. D-group chromosomes (Nos. 13, 14, and 15) from normal and hyperdiploid cell lines. Top row, G-banded; middle row, C-banded; bottom row, Q-banded. Left side, normal (46, XX) cell line; right side, hyperdiploid cell line. Q-banded row includes No. 21 chromosome pair for comparison of its banding properties with those of the 15p+ region. Arrows indicate polymorphic variant 15p+.

every cell; this arm was dark on G-banding, pale on Q-banding, and dark on C-banding (Fig 1). On the basis of these staining properties, it was interpreted as a polymorphic variant. The second population in both tissues was hyperdiploid, with tetrasomy for chromosomes 8, 10, and 21 (Fig 2). In these cells, *both* No. 15 chromosomes had the large, dark-staining p arm (Fig 1). The karyotype was described as: 46, XX, var(15) (p11.2, QFQ42, CBG40)/52, XX, +8, +8, +10, +10, +21, +21, var(15 = 2) (p11.2, QFQ42, CBG40).

Sample 3 was peripheral blood taken ten days after the start of chemotherapy. Only five metaphases could be found; all had a normal female karyotype with the polymorphic variant 15 found once per cell.

Sample 4, a bone marrow specimen taken 17 days after the start of therapy, had no mitotic activity.

Sample 5, another bone marrow taken 35 days after the start of therapy and 8 days after its conclusion, showed a normal female karyotype, with the variant 15 found once per cell, in 19 of 20 cells examined. One cell was tetraploid.

DISCUSSION

The presence of many chromosomally normal cells along with a chromosomally abnormal, malignant clone is a common finding in leukemic disorders.[2] Both populations may appear in mitogen-stimulated blood as well as in marrow; the malignant clone will disappear after successful therapy. The abnormal clone found in the pretreatment blood and bone marrow of our patient had two types of cytogenetic abnormalities. This clone disappeared from both blood and marrow after chemotherapy. One abnormality, the finding of hyperdiploidy, is not surprising insofar as it has often been reported in patients with acute leukemia, both ALL and AML.[2,3] Gains of chromosomes 8 and 21 are common in AML. Gain of 21 is also common in childhood ALL; gains of 8 and 10 are less common in that disease but have been observed.[2,3]

The second type of cytogenetic abnormality observed in our patient is an unusual structural change: A polymorphic variant, present on one No. 15 chromosome in the normal cell line, was found on both No. 15 chromosomes in the malignant hyperdiploid clone. Several mechanisms could account for the change from heterozygosity to homozygosity for this region.

First, the change could be due to simultaneous loss of the nonvariant 15 and duplication of the variant 15, leading to homozygosity for both arms, ie, for the entire chromosome. This could be caused by double nondisjunction of chromatids at a mitotic division.

Somatic recombination is a second mechanism that could lead to homozygosity for 15p+. Crossing over just above the centromere, for example, could lead, in the next cell generation, to a cell line in which both No. 15s have the large polymorphism. However, in this case, the remainder of the chromosomes (the 15q arms) would remain unchanged and any genetic heterozygosity present there originally would be retained. Somatic, or mitotic, recombination is a rare but well-documented phenomenon[4] that has been known for years in *Drosophila* and in yeast and other fungi. Cancer geneticists have recently turned their attention to this phenomenon because of the discovery that retinoblastoma tissue often shows homozygosity for markers on chromosome 13 that were heterozygous in the patient's unaffected cells.[5]

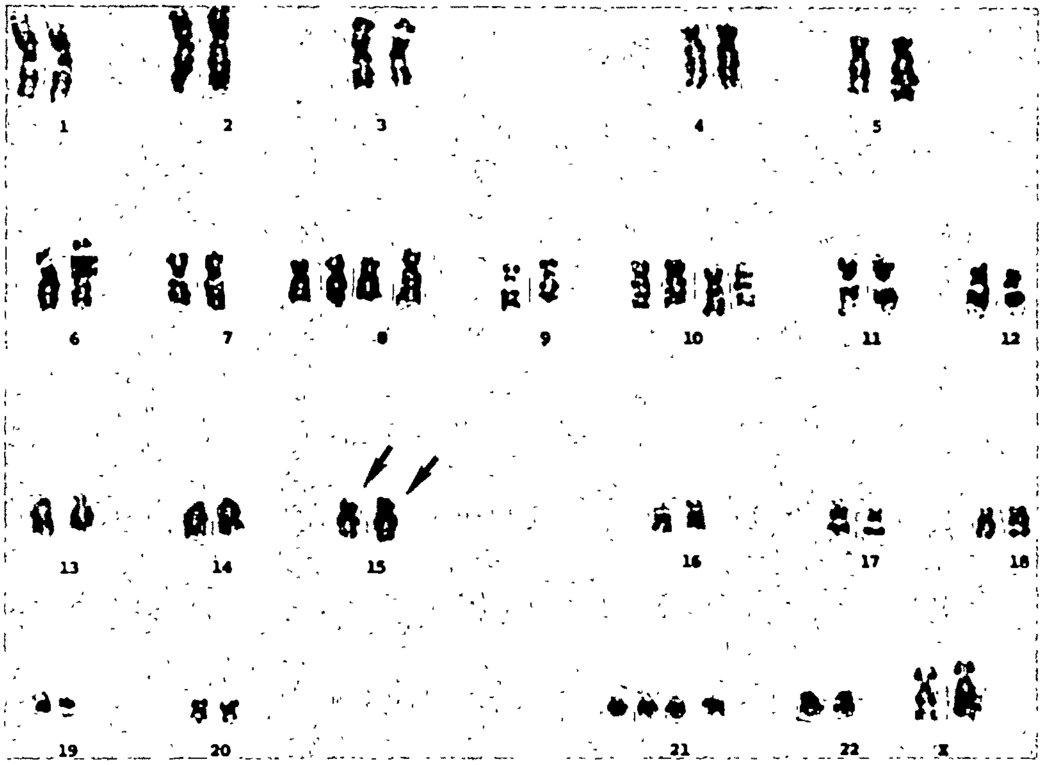

Fig 2. Hyperdiploid karyotype found in pretreatment blood and bone marrow: 52, XX, +8, +8, +10, +10, +21, +21. Arrows indicate polymorphic variant on short arm of both No. 15 chromosomes.

Wilms' tumor tissue often exhibits a similar shift to homozygosity for at least a part of chromosome 11.[6,7] In retinoblastoma, the shift is apparently due to mitotic nondisjunction in some cases and to mitotic recombination in others; in Wilms' tumor, the mechanism is not yet known. However, it now seems likely that one or both of these genetic events is often crucial to the development of tumors.

In the case of retinoblastoma and Wilms' tumor, deletions of the chromosomal regions where homozygosity has been demonstrated (13q14 and 11p13, respectively) are known to be associated with development of the tumors.[8] In the present instance, our patient was diagnosed as having ALL, and homozygosity at least for 15p (and possibly all of chromosome 15) was demonstrated cytogenetically. The relation between these two phenomena is not clear and need not be causal. Deletions of a region of chromosome 15, or of the entire chromosome, have been described in some cases of ALL, but it is not an especially frequent finding.[2,3,9] In addition, the area of chromosome 15 where homozygosity is cytogenetically demonstrable (15p) is an area where deletions and duplications are tolerated and have no clinical consequence[10]; the definition of the 15p+ as a polymorphic variant bears this out. Band 15q22, on the long arm, is notable for its consistent involvement in a 15;17 translocation in acute nonlymphocytic leukemia type M3[9]; in addition, the feline sarcoma (*fes*) oncogene is located on band 15q24–25 on the long arm of chromosome 15.[8] The shift to homozygosity that we observed in 15p may be the visible manifestation of a shift to homozygosity for the entire No. 15 chromosome. If the two No. 15s had different alleles for *fes*, the shift would have eliminated one allele and doubled the other. *Fes* is known to be highly expressed in both ALL and AML, whereas there is no evidence of *fes* transcripts in normal cells.[11]

Alternatively, it may be that random mitotic nondisjunction and/or mitotic recombination, both leading to homozygosity for at least part of a chromosome, are not uncommon events in malignant cells. Just as apparently random gain or loss of chromosomes in AML and ALL indicates a general disturbance of normal segregation mechanisms, so may the processes leading to homozygosity of apparently random chromosomes indicate a general genetic instability in these cells. Observation of chance chromosomal polymorphisms, as in this case, combined with the use of DNA polymorphisms, should provide insight into these events.

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Influence of the Calcium-Sensitive Fluorophore, Quin 2, on Platelet Function

By Gundu H.R. Rao, Janet D. Peller, Charles P. Semba, and James G. White

Recent investigations using Quin 2, a fluorophore used to monitor cytosolic free calcium shifts, have shown that strong agonists cause a dramatic dose-dependent increase in platelet fluorescence. However, weak agonists stimulated little or no increase in light emission of Quin 2- loaded platelets, suggesting that calcium flux is not involved in activation by these agents. The present study has sought an alternative explanation for the failure of weak stimuli to cause a rise in cytosolic free calcium in platelets containing Quin 2. Conditions used to prepare, wash, load, gel-filter, and evaluate the fluorophore-filled cells were examined for their compatibility with retention of sensitivity to activation by weak agonists. The technique used to measure shifts in cytosolic calcium with Quin 2 requires multiply washed, unstirred platelets. Under these conditions, platelets do not aggregate or secrete in response to weak agonists. Quin 2, at concentrations >40 μmol/L, inhibits the response of platelets to strong agonists, and completely blocks their reaction to weak agonists. Quin 2 inhibition of platelet function appears related to high buffering capacity for free calcium, although other mechanisms cannot be ruled out. This suggestion is supported by the observation that Quin 2-induced blockade can be overcome by membrane modulation, which is a calcium-dependent process. However, since both agonists are weak, significant elevation in cytosolic calcium concurrent with functional restoration could not be demonstrated under the experimental conditions used for monitoring calcium. Thus, the conditions used to prepare platelets for Quin 2 evaluation and Quin 2 itself appear to be responsible for the failure of weak agonists to cause evidence of a calcium shift in fluorophore-loaded cells.

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UNSTIMULATED blood platelets have a characteristic discoid form in vivo and in vitro.[1,2] Following exposure to a variety of chemical agonists and foreign surfaces, platelets lose their lentiform appearance, extend pseudopods, undergo internal transformation, and contract.[3,4] The nature of the physical changes has suggested that platelets are an unusual type of muscle cell, and contractile physiology dominates their response to nearly all stimuli.[5-7]

Calcium movement from the sarcoplasmic reticulum to the cytoplasm is the final common pathway for triggering contraction in muscle systems.[8-10] It seemed reasonable, therefore, to suggest that calcium flux may serve a similar role in activation of platelet contractile activities.[11] Many studies supporting this concept have provided evidence that calcium movement into platelets triggers the same sequence of contractile phenomena observed in the physiological reactions to aggregating agents.[12-15]

Introduction of a calcium-sensitive fluorophore, Quin 2, has made it possible to demonstrate directly a rise in platelet cytoplasmic calcium in response to agonists.[16-19] The esterified form of the agent renders it soluble in membranes. Esterases in the cell surface cleave the ester bond, permitting the water-soluble form of Quin 2 to concentrate in platelet cytoplasm.[20] Exposure of Quin 2-loaded platelets to potent agents such as thrombin or ionomycin results in a dramatic, dose-dependent increase in fluorescence.[17]

However, the response of platelets filled with the fluorophore to weak agonists has been unimpressive.[19,21-23] Serotonin, adenosine diphosphate (ADP), and epinephrine failed to cause a significant increase in fluorescence of Quin 2-loaded cells. The results have suggested that calcium flux is not involved in platelet activation by these agents and has led many workers to search for a non–calcium-dependent pathway.[24-27]

The purpose of the present investigation was to determine if there might be an alternative explanation for the failure of Quin 2-loaded platelets to fluoresce following stimulation by weak agonists. Conditions used to prepare, load, wash, and evaluate the fluorophore-filled cells were examined for their compatibility with retention of sensitivity to activation by weak agonists. In addition, we have examined the influence of Quin 2 as an inhibitor of platelet aggregation. Results of these studies suggest that the importance of calcium flux as an initial step in the response of platelets to activation by weak agonists is not ruled out by their failure to stimulate fluorescence in Quin 2-loaded cells.

MATERIALS AND METHODS

G-^{3}H-Quin 2, tetraacetoxymethyl ester (Quin-2-AM, 5 to 15 mCi/mmol), was purchased from Amersham, Arlington Heights, Ill, and nonradioactive Quin-2-AM and the free acid was purchased from Lancaster Synthesis Ltd, England. Prostaglandin E_1, prostacyclin, and endoperoxide analog (U46619) were gifts from the Upjohn Co, Kalamazoo, Mich. 1-^{14}C-arachidonic acid was obtained from New England Nuclear, Boston. Arachidonic acid as the sodium salt was purchased from NuChek Prep, Elysian, Minn, and was made up in 0.1 mol/L of Tris buffer, pH 7.40. Injectable adrenaline and bovine thrombin were from Parke-Davis Co, Detroit. Platelet-activating factor was a gift from Professor Harold H. O. Schmid of the Hormel Institute, Austin, Minn. Calcium ionophore A23187 was from Calbiochem/Behring, Los Angeles. Unless otherwise mentioned, all other chemicals were obtained from Sigma Chemical Co, St Louis.

Platelet preparation and measurement of cytosolic calcium. Blood was drawn from adult volunteers who had not taken any medications for at least two weeks prior to venesection and was mixed immediately with citrate-citric acid-dextrose (CCD) anticoagulant (citrate, 0.1 mol/L; citric acid, 7 mmol/L; dextrose, 0.14

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mol/L, pH 6.5) in a ratio of nine parts blood to one part anticoagulant.[28] Platelet-rich plasma (PRP) was obtained by centrifugation of whole blood at room temperature for 20 minutes at 100 g, and the platelet count was adjusted to 300,000/μL with platelet-poor plasma. Platelet aggregation studies were carried out on a Payton dual-channel aggregometer preset with PRP and platelet-poor plasma.[29] Release of ATP was monitored using a Chronolog lumiaggregometer.[30]

Purity of the nonradioactive Quin-2-AM was checked by analyzing the stock material by high-pressure liquid chromatography (HPLC).[31] The Waters analytical model HPLC with a variable wavelength detector was used for these studies. A radial-pack C18 reverse-phase column was used for the separation of Quin-2-AM from the free acid. The solvent system consisted of 80% methanol:water, and the compounds were eluted at a flow rate of 2.0 mL/min monitored at 254 nm. ^3H-Quin-2-AM was used for cochromatography with the nonradioactive compound. Peaks representing Quin-2-AM were collected and counted for confirmation of the authenticity of the compound.

Radiolabeled Quin-2-AM uptake was studied using platelet-rich plasma. One-milliliter samples of PRP were incubated at room temperature for 1, 5, 10, and 60 minutes. Samples were sedimented (Beckman microfuge) for one to five minutes with EDTA added to prevent activation. After the supernatant was transferred to counting vials, platelet samples were resuspended in 1 mL of CCD and sedimented again for one to five minutes. After the supernatant was separated, platelet pellets and supernatant fractions were analyzed for radioactivity.

For evaluation of the effect of processing (washing/gel filtering) on the functional capability, platelets were incubated with 20 μmol/L of Quin 2 for ten minutes at 37 °C and then washed with either the CCD anticoagulant solution (1:1 vol/vol) or EDTA (1:10 vol/vol, pH 7.4, 0.34 mol/L). Some samples were processed with a combination of EDTA (1:10 vol/vol, 0.34 mol/L) and PGE_1 (1 μmol/L, 37 °C, ten minutes). EDTA-washed platelets were prepared by two different methods: (1) a fast two-minute sedimentation in a microfuge (Beckman) at 8,740 g, or (2) a 20-minute centrifugation at 1,660 g in a refrigerated centrifuge. Finally, platelets were gel-filtered on sepharose-2B columns. All preparations were resuspended in HEPES buffer, platelet counts were adjusted to 300,000/μL, and preparations were tested for their response to agonists.

In order to evaluate the inhibitory effect of Quin 2 on platelet function, cells were loaded with varying concentrations of Quin 2 (20 to 100 μmol/L) at room temperature and exposed to the action of agonists on an aggregometer. For measurement of intracellular calcium, Quin 2-loaded platelets (20 μmol/L, ten minutes at 37 °C) were washed, gel-filtered, and resuspended in HEPES buffer containing 145 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L of $MgSO_4$, 0.5 mmol/L of Na_2HPO_4, 1 mmol/L of $CaCl_2$, 10 mmol/L of HEPES, 5 mmol/L of glucose (pH 7.4).[20] Fluorescence measurements were performed with a Fluorolog scanning photon-counting spectrofluorometer. Approximately 4×10^8 cells (2 mL) were used for these studies. Agonists and antagonists were added to platelets in a cuvette, mixed gently, and scanned for their fluorescence spectra. Excitation and emission wavelengths were 339 and 495 nm, respectively.[20] Intracellular Quin 2 concentrations were determined by comparing the Ca^{2+}-induced fluorescence of Quin 2-loaded platelets which had been lysed with 0.1% Triton (Sigma Chemical Co, St Louis) with the fluorescence of a standard solution of Quin 2 free acid in the presence of unloaded cells treated with 0.1% Triton and calcium. Measurements of cytosolic calcium were performed 30 minutes after restoration of extracellular calcium. The maximal fluorescence (F_{max}) was determined after lysing platelets with Triton in the presence of 10 mmol/L of calcium. Minimal fluorescence (F_{min}) was determined by lysing platelets with Triton in the presence of 4 mmol/L of EGTA at a pH of 8.5. The intracellular free calcium was calculated according to the formula $(Ca^{2+})_i = 115(F - F_{min})/(F_{max} - F)$.[20]

To follow the effect of Quin 2 and the influence of various agonists on Quin 2-loaded platelets, control and treated samples were fixed for study in the electron microscope according to the procedures reported in detail from this laboratory.[4] Platelets exposed to the highest concentration of Quin 2 (80 μmol/L) were studied for serotonin and adenine nucleotide content and their ability to convert ^{14}C-arachidonic acid to thromboxane by the methods described previously.[32,33]

To measure ^{14}C-arachidonic acid conversion to thromboxane by intact platelets, each reaction mixture containing 1.5×10^9 cells suspended in 1 mL of Hank's Balanced Salt Solution (HBSS) was stirred on an aggregometer for five minutes at 37 °C with 1 μg of labeled arachidonic acid.[34] For evaluating the effect of Quin-2-AM, the platelets exposed to various concentrations of the drug for different incubation periods were used. At the end of the experiment, 1 mL of ethyl acetate was added to each reaction mixture and acidified to pH 3.5 with 0.5 mol/L of citric acid. After thorough mixing, the ethyl acetate layer was separated, and the samples were reextracted with an equal volume of ethyl acetate. Pooled ethyl acetate extract was concentrated with nitrogen and spotted on a thin-layer plate (silica gel G). The solvent system used for the separation of thromboxane was ether:methanol:acetic acid (135:2:2 vol/vol). Radioactive metabolites were monitored with a Berthold radiolabel scanner and quantitation was achieved by scraping radioactive spots off the thin-layer plate and scintillation counting.

All aggregation studies were repeated at least five times with platelets obtained from different donors. Tracings presented represent a typical response obtained under the experimental conditions. Values for biochemical studies are expressed as mean ± SE. Statistical analysis was performed using unpaired Student's t test.

RESULTS

Quin 2 stability during storage. The quality of Quin 2 and the presence of contaminants may influence the response of fluorophore-loaded platelets to stimulation. Therefore, the purity and reactivity of stock Quin 2 were checked periodically by HPLC. Control studies made over a period of several months showed that Quin 2 suspended in dimethyl sulfoxide and stored at −35 °C remained stable. The product used in these studies did not have any major ultraviolet (UV)-absorbing contaminants.

Uptake of Quin 2 by platelets in plasma. Studies were conducted with radiolabeled ^3H-Quin 2 to evaluate the kinetics of uptake. It was found that ten minutes of incubation at 37 °C with Quin 2 at 20 μmol/L was adequate to achieve optimum concentrations of the dye in platelets. However, for elevating intracellular concentrations to >2 mmol/L, either incubation with higher concentrations (40 to 60 μmol/L) of the dye or longer periods of exposure to lesser amounts (20 to 40 μmol/L) was essential.

Influence of time on the basal level of calcium measured. The basal level of measured calcium is critical for computing any alterations due to pathological, physiological or pharmacological conditions. Therefore, we explored the influence of the length of exposure of platelets to Quin 2 on the apparent basal value of calcium detected by Quin 2. Basal values changed gradually during prolonged exposure to the drug. As shown in Fig 1, the initial level of cytoplasmic calcium measured on three identical samples (70 ± 11 nmol/

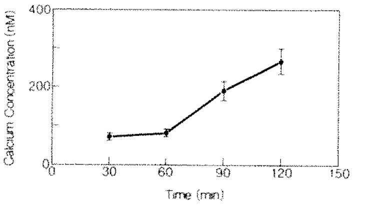

Fig 1. The basal level of calcium in resting platelets was followed over a period of two hours by monitoring changes in Quin 2 fluorescence. With the increase in time, there was a significant elevation in the basal level of calcium in unactivated platelets. Values presented are mean and SE for four replicates.

L) increased to 188 ± 22 nmol/L by 90 minutes and to 262 ± 28 nmol/L by 120 minutes. Therefore, the basal level of calcium was measured before and after each experiment in this study.

Agonist-induced elevation of cytosolic calcium. Resting platelets monitored within the first hour of sample preparation had ~100 ± 6 nmol/L of calcium (n = 12). Exposure of these platelets to concentrations of thrombin greater than 0.2 U/mL elevated cytosolic calcium levels above 1 μmol/L (Fig 2). Addition of prostacyclin (10 ng/mL) or PGE_1 (1 μmol/L) lowered calcium to the basal level or lower (Table 1). Addition of these agents to platelets before exposure to thrombin significantly inhibited the thrombin-induced rise of calcium. Although other agonists such as epinephrine, ADP, collagen, arachidonate, platelet-activation factor, and endoperoxide analog induced some degree of increase in cytosolic calcium, the results were neither consistent nor statistically significant.

Effect of processing Quin 2-loaded platelets on the functional capability of platelets. Washed/gel-filtered platelets suspended in HEPES did not aggregate or secrete when stirred with epinephrine (5 μmol/L), ADP (3 μmol/L), or serotonin (5 μmol/L). EDTA-washed platelets resuspended in buffered HEPES responded poorly to threshold concentrations (3 to 10 μmol/L) of arachidonate. However, at higher concentrations (> 10 μmol/L), arachidonate induced secretion of ATP and caused aggregation. On the other hand, thrombin, in as low a concentration as 0.1 U/mL, induced significant release of ATP. Release of ATP induced by 0.1 U of thrombin was greater than that caused by 20 μmol/L of arachidonate. Not only did EDTA-washed platelets respond poorly to arachidonate, but platelets exposed to prostacyclin or PGE_1 were also refractory to the action of arachidonate. However, the PGI_2-treated or PGE_1-treated platelets did aggregate and secrete ATP when exposed to thrombin.

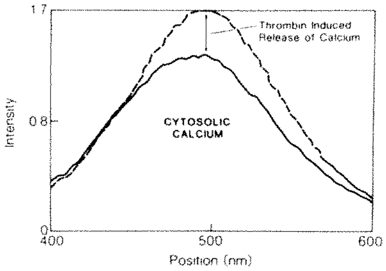

Fig 2. Fluorescence was monitored in normal, washed human platelets loaded with Quin 2 before and after stimulation with thrombin (0.5 U/mL) in the presence of 1 mmol/L of calcium. Increased fluorescence was observed (upper tracing) immediately following thrombin addition.

Table 1. Influence of PGI_2 (100 nmol/PGE_1 (1 μmol/L) on Cytosolic Calcium Levels

	Calcium (nmol/L)*
Resting platelets	96 ± 12
Platelets + thrombin (0.5 U/mL)	↑1000†
Platelets + (PGI_2/PGE_1) + thrombin	92 ± 8
Platelets + thrombin + (PGI_2/PGE_1)	86 ± 10

Influence of prostacyclin (PGI_2) and E_1 (PGE_1) on thrombin-induced alterations in cytosolic calcium of Quin 2-loaded platelets was followed. Addition of these agents to platelets prior to thrombin exposure blocked agonist-induced elevation of calcium. Exposure of platelets to these compounds following thrombin-induced activation resulted in lowering of calcium to basal levels.

*Mean and SE (n = 3).

†Thrombin-induced increase was >1,000 nmol/L of calcium in all studies.

Stirring at a constant speed is important for obtaining maximum secretion and irreversible aggregation. The effect of stirring (1,000 rpm) on the response of platelets to agonists was explored using Quin 2-loaded (20 μmol/L) platelets in plasma as well as in buffered HEPES. Thrombin and arachidonate induced significant release of ATP in unstirred platelets in plasma. Weak agonists, such as epinephrine, ADP, and serotonin, did not induce secretion or aggregation in unstirred platelets in plasma. Quin 2-loaded platelets suspended in buffered HEPES responded poorly to arachidonate whether the samples were stirred or not. Only thrombin could induce significant release of ATP in unstirred, washed platelets suspended in buffered HEPES. Quin 2-loaded platelets released comparatively less ATP when challenged with 0.1 U/mL of thrombin than did control platelets.

Effect of incubation with Quin 2 on morphology and biochemistry. Platelets incubated with concentrations of Quin 2 as high as 80 μmol/L for up to 60 minutes retained normal levels of serotonin and adenine nucleotides (Table 2). Accumulation of the fluorophore under these conditions had no apparent effect on platelet morphology. Most cells retained their discoid form and signs of activation, such as pseudopod formation, internal contraction, and disappearance of dense bodies and α granules, were not evident (Fig 3).

Effect of Quin 2 on agonist-induced platelet aggregation. Normal platelets in plasma, when stirred with epinephrine (5 μmol/L), adenosine diphosphate (3 μmol/L), arachidonate (0.45 mmol/L), and thrombin (0.2 U/mL), aggregated and released significant quantities of ATP. However, exposure of platelets to Quin 2 for 20 minutes at room temperature at concentrations >40 μmol/L induced severe dysfunction. Quin 2 exerted a dose-dependent inhibition of arachidonate-induced aggregation (Fig 4), and impaired the release reaction as well (Fig 5). At 80 μmol/L concentration, Quin 2 totally abolished the response of platelets to the action of all agonists, including thrombin, the calcium ionophore A23187, the endoperoxide analog U46619, and platelet-activating factor.

Table 2. Effect of Quin 2 (80 μmol/L) on Platelet Serotonin and Total Adenine Nucleotides

	Control Platelets	Treated Platelets
Serotonin (ng/10^9 cells)	741 ± 64	873 ± 56
Adenine nucleotides (μmol/10^{11} cells)		
AMP	0.6 ± 0.08	0.8 ± 0.08
ADP	3.5 ± 0.2	3.0 ± 0.2
ATP	4.9 ± 0.2	4.6 ± 0.2

Mean and SE (n = 4).

The effect of Quin 2 on platelet serotonin and adenine nucleotide levels was followed. Exposure of platelets to as high as 80 μmol/L concentration of Quin 2 did not induce any alterations in the levels of serotonin and adenine nucleotides.

Effect of Quin 2 on platelet cyclooxygenase activity. Because Quin 2 inhibited arachidonate-induced aggregation and the release reaction, its effect on arachidonic acid conversion to thromboxane was followed. Normal platelets converted radiolabeled arachidonic acid to thromboxane (Table 3). Quin 2-treated platelets did not show any significant inhibition in their ability to convert radiolabeled arachidonic acid to thromboxane, suggesting that Quin 2-induced inhibition is not related to inhibition of cyclooxygenase activity.

Reversal of Quin 2 inhibition. To see if Quin 2-induced inhibition of platelet function was reversible, Quin 2-loaded platelets were washed and resuspended in fresh drug-free plasma and challenged with arachidonate, Quin 2-loaded platelets in fresh plasma did not aggregate when stirred with arachidonate, suggesting that the fluorophore present in the cytosol is still capable of exerting an inhibitory effect.

Earlier studies have demonstrated that the mechanism of membrane modulation can restore the sensitivity of refractory platelets to the action of agonists.[34-36] Quin 2-treated platelets (40 μmol/L) did not aggregate when stirred with epinephrine (5 μmol/L) or arachidonate (0.45 mmol/L). However, on exposure to epinephrine, the refractory cells regained their sensitivity and aggregated irreversibly to a subsequent challenge with arachidonate (Fig 6).

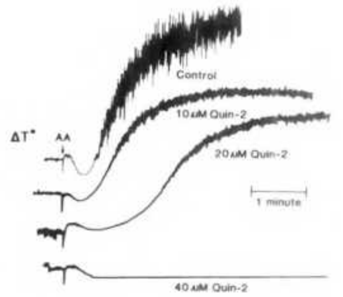

Fig 4. Effect of different concentrations of Quin 2 on (0.45 mmol/L) arachidonate-induced platelet aggregation was followed on an aggregometer. Quin 2 exhibited a dose-dependent inhibition on the ability of arachidonate to cause platelet aggregation. *ΔT, change in light transmission.

Influence of epinephrine on the ability of refractory platelets to mobilize calcium. The ability of epinephrine to restore sensitivity to platelets made refractory by Quin 2 may have been due to restoration of a rise in cytoplasmic calcium to significant levels. The refractory model examined to test this hypothesis was described previously.[37] ADP-induced aggregates were dissociated with PGI_2. Prostacyclin-dissociated cells are refractory to the action of all agonists, but an initial exposure to epinephrine restores the sensitivity of these refractory platelets to the action of all agonists. When normal cells were loaded with Quin 2, the addition of ADP did not cause any elevation in cytosolic calcium levels (Fig 7). Exposure of ADP-treated platelets to prostacyclin induced a significant drop in cytosolic calcium. Addition of epinephrine alone or thrombin alone to PGI_2-dissociated cells did not elevate calcium above basal levels. However, exposure of PGI_2-treated refractory platelets to epinephrine first, followed by thrombin, resulted in a maximum rise of cytosolic calcium.

DISCUSSION

The present investigation has tried to determine why weak agonists capable of causing irreversible platelet aggregation and secretion have reportedly failed to cause an increase in cytoplasmic calcium as measured by the fluorophore, Quin

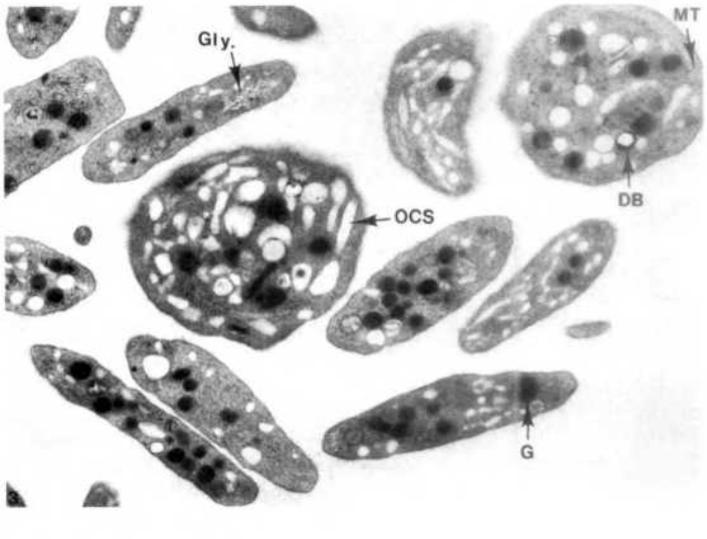

Fig 3. Platelets from a sample of platelet-rich plasma incubated with 80 μmol/L of Quin 2 for 60 minutes at 37 °C. These platelets have retained their characteristic discoid shape. Microtubules (MT) are present just beneath the cell membrane as in resting cells. Dense bodies (DB), granules (G), and glycogen masses (Gly) are scattered randomly in the platelet interior. Signs of cell activation, shape change, pseudopod formation, and centralization of granule contents are not evident in these treated platelets. Original magnification ×13,000; current magnification ×8,450.

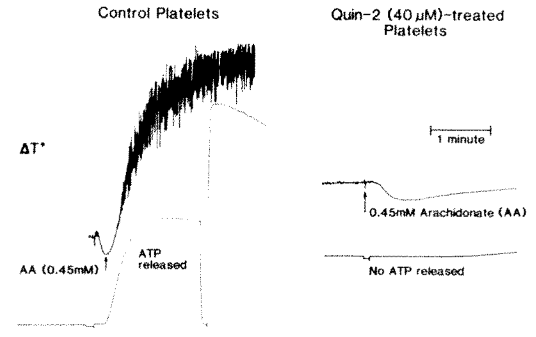

Fig 5. Effect of Quin 2 on platelet secretion and irreversible aggregation was followed. At 40 μmol/L concentration, Quin 2 effectively blocked ATP secretion as well as irreversible aggregation induced by arachidonate (0.45 mmol/L). *ΔT, change in light transmission.

2.$^{21-23}$ Our initial experiments in this study confirmed the observations of other workers. Potent agonists, such as thrombin, the endoperoxide analog U46619, platelet-activating factor, and the calcium ionophore A23187, cause a significant increase in fluorescence of Quin 2-loaded platelets. In addition, agents that stimulate adenylate cyclase, including prostacyclin (PGI_2), PGE_1, and forskolin, were shown to prevent the increase in Quin 2 fluorescence caused by potent agents and to reduce elevated levels of calcium stimulated by prior exposure to strong agonists. Weak agonists, such as epinephrine, serotonin, or ADP, failed to produce any consistent rise in cytosolic calcium levels, as noted previously.[22,23]

The inability of the weak stimuli to generate increased levels of calcium could be related to the procedures used to load platelets with Quin 2 and measure changes in fluorescence. Initially, we evaluated the stability of the drug in storage as well as the influence of the carrier solvent. Quin 2 is made up and stored in dimethyl sulfoxide (DMSO). Analysis of the stock solution over the period of this study revealed no degradation of the drug during storage. Although DMSO itself can inhibit platelet function, care was taken not to add more than 10 μL of DMSO per reaction in these studies.[12] Where needed, appropriate controls were used with the carrier solvent alone. Results of our studies demonstrate that neither the degradation products nor the carrier solvent was responsible for the failure of weak agonists to stimulate fluorescence of Quin 2 loaded platelets.

Table 3. Influence of Quin 2 (80 μmol/L) on 1-^{14}C-Arachidonic Acid Conversion to Thromboxane (T × B_2) by Human Platelets

	CPM × 10^3		
	Total Radioactivity Recovered	Counts as T × B_2	Conversion to T × B_2 (%)
Control platelets	126.4 ± 6.9	34.6 ± 3.8	27.3 ± 2.4
Platelets in plasma			
Quin 2 (80 μmol/L)	138.2 ± 7.8	36.8 ± 3.2	26.6 ± 3.2
Washed platelets			
Quin 2 (80 μmol/L)	118.6 ± 5.1	32.4 ± 2.8	27.3 ± 2.6

Mean and SE (n = 4).

Influence of Quin 2 on the ability of platelets to convert arachidonic acid to thromboxane was followed. Quin 2 at the inhibitory concentration (80 μmol/L) did not block platelet cyclooxygenase. Quin 2-treated platelets converted as much of arachidonic acid to thromboxane as did normal control platelets.

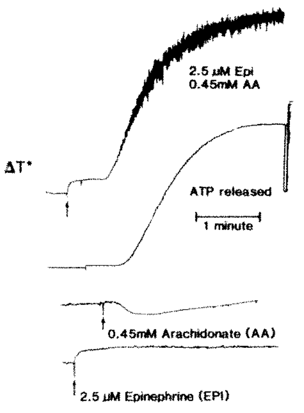

Fig 6. The ability of epinephrine-induced membrane modulation to restore function in Quin 2-loaded, arachidonate-refractory platelets was followed. At 40 μmol/L concentration, Quin 2 inhibited arachidonate-induced aggregation as well as epinephrine-induced secondary wave. However, refractory platelets, on exposure to epinephrine, regained their sensitivity and aggregated irreversibly when challenged with arachidonate. *ΔT, change in light transmission.

Preparation of platelets for Quin 2 studies involves removal of plasma and the unreacted drug by washing or gel-filtering through a sepharose column.[17-19] Processing of platelets in this way may induce activation and, subsequently, a refractory state. Several methods commonly used were evaluated to see if any of them induced inhibition of platelet function.[17-19,27] Quin 2-loaded, washed, or gel-filtered platelets resuspended in buffered HEPES did not aggregate or secrete when stirred with weak agonists. As a result, it is unlikely that weak agonists could stimulate a calcium flux in the nonresponsive platelets.

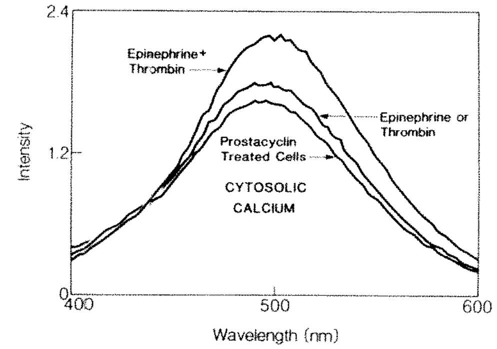

Fig 7. Agonist-induced and antagonist-induced alterations in cytosolic free calcium levels were monitored by measuring Quin 2 fluorescence. ADP did not induce any alterations in the basal level of calcium. Prostacyclin induced a significant drop in the calcium level. Epinephrine used alone was unable to restore the calcium concentration to the basal level. Thrombin restored the calcium to basal level, but was unable to elevate it further. However, exposure of prostacyclin-treated platelets first to epinephrine and then a challenge by thrombin gave the maximum elevation of cytosolic calcium.

The response of washed platelets to arachidonate was also relatively poor. Higher concentrations of arachidonate (20 μmol/L and above) induced aggregation and limited amounts of secretion. Only thrombin induced secretion in Quin 2-loaded platelets suspended in buffered HEPES. Because aggregation of platelets interferes with fluorescence monitoring, all calcium measurements were made using unstirred platelets.[18] Thrombin and high concentrations of arachidonate induced secretion in unstirred, washed platelets. Weak agonists such as epinephrine, ADP, or serotonin did not induce any secretion in unstirred, Quin 2-loaded, washed platelets. Therefore, it is reasonable to suggest that the conditions used may cause a refractory state and prevent weak agonists from eliciting elevation of cytosolic calcium.

Although arachidonate can induce release of ATP in unstirred platelets, the concentrations required to achieve significant secretion were high. Washed platelets are easily lysed by high concentrations of fatty acids.[38] Any lysis of platelets during calcium measurement will add significantly to the values obtained following exposure to agonists. It is difficult to detect partial lysis under the experimental conditions used, and therefore hard to select an optimum level of arachidonate that will elicit significant elevation of cytosolic calcium without inducing platelet lysis. Even though arachidonate could induce secretion of unstirred, washed platelets, our studies did not provide statistically significant data that it does so under the conditions required in Quin 2 investigations.

Prostacyclin or PGE_1 are frequently included in Quin 2 preparative procedures to prevent activation of platelets. Although these drugs are removed during washing or gel-filtering, the inhibitory effect may persist. Sinha and Colman have shown that continued presence of the hormone (PGE_1) is not essential for the maintenance of elevated levels of cAMP.[39] Therefore, it is reasonable to suggest that the elevated intracellular levels of cAMP will induce a refractory state in PGE_1/PGI_2-exposed platelets. In this study, we evaluated the effect of including PGE_1 (1 μmol/L) in the washing procedure on the response of washed platelets to arachidonate and thrombin, the two agonists that were capable of inducing secretion in unstirred platelets. PGE_1-treated platelets did not respond to arachidonate, although thrombin could still elicit significant release of ATP. Therefore, if Quin 2 studies involve evaluation of the response of platelets to the action of weak agonists, use of drugs that stimulate adenylate cyclase in the washing procedures may block the platelet response and prevent fluorescence secondary to calcium flux.

Elevation of cytosolic free calcium has been considered the final common pathway for achieving platelet activation. Quin 2 has a high buffering capacity for calcium. By complexing small amounts of calcium released into the platelet cytoplasm following exposure to weak agonists, Quin 2 may block platelet calcium requiring functions.[19,40-42] At low concentrations (>20 μmol/L), the fluorophore did not inhibit platelet aggregation stimulated by weak agonists. However, at concentrations of ≥40 μmol/L, Quin 2 blocked the aggregation response to all physiological agents. It also inhibited formation of aggregates caused by the ionophore A23187, the endoperoxide analog U46619, and platelet-activating factor. Thus, Quin 2 is a powerful antagonist of platelet activation, either through its ability to buffer calcium released into platelet cytoplasm or through some other mechanism.[43]

Other investigators, using platelets and neutrophils, have shown that Quin 2 can inhibit cell function.[40-42] Affolter et al have demonstrated that serotonin and A23187 induced platelet shape change are prevented by Quin 2.[40] Johnson et al have found that the release reaction is inhibited in Quin 2-loaded platelets.[19] Lew and co-workers have reported that the calcium rise measured by fluorescence and extent of granule release in neutrophils correlated with the concentration of Quin 2 loaded into the cells.[41] Quin 2 must be present in a sufficient concentration inside the cells (>1 mmol/L) to provide a detectable fluorescence signal on complexing with calcium.[19,44] As a result, it is difficult to select a concentration of Quin 2 to achieve satisfactory calcium measurements that does not, at the same time, interfere with agonist-induced activation, secretion, and aggregation. It is reasonable to suggest that the buffering capacity of Quin 2 in amounts necessary for detecting calcium flux can prevent stimulation and significant calcium flux by weak aggregating agents.

Several studies from our laboratory have demonstrated the ability of epinephrine to restore the sensitivity of refractory platelets to the action of agonists.[34-39] Therefore, we explored the possibility of restoring the sensitivity of platelets made refractory by Quin 2 to the action of agonists by epinephrine-induced membrane modulation. Using the well-characterized model of the aggregation–disaggregation–reaggregation cycle, we evaluated the ability of epinephrine to facilitate calcium mobilization.[37] Although epinephrine and thrombin alone did not induce elevation of cytosolic free calcium, epinephrine facilitated calcium mobilization by thrombin in cells dissociated by PGI_2 from ADP aggregates. Restoration of sensitivity to refractory platelets by epinephrine has been related previously to its ability to modulate α-adrenergic receptors and facilitate calcium mobilization by agonists.[29,30,34-37] Results of the present study support the concept that weak agonists activate platelets through calcium-dependent pathways that are blocked by loading the cells with Quin 2.

In conclusion, the present investigation provides an explanation of why weak agonists do not cause significant elevation of cytosolic calcium. Calcium measurement with Quin 2 is done using washed platelets in unstirred conditions. Weak agonists, such as epinephrine, ADP, or serotonin, do not induce secretion in unstirred, washed platelets. In view of these findings, it is reasonable to suggest that weak agonists induce platelet activation through calcium mobilization, but limitations imposed by the techniques of Quin 2 loading or Quin 2 itself prevent demonstration of such a mechanism. On the other hand, thrombin readily induces secretion in unstirred, washed platelets and is capable of causing a significant elevation of cytosolic calcium. Quin 2 at >40 μmol/L concentration inhibits the response of platelets to all physiological agonists. However, epinephrine-induced α-adrenergic receptor stimulation restores the sensitivity of Quin 2-loaded, refractory platelets to the action of agonists by facilitating calcium mobilization. Results of the investigation indicate that the conditions used in Quin 2 studies are most likely responsible for failure of weak agonists to cause a detectable calcium flux. Lack of a burst of fluorescence in response to stimulation by weak agonists cannot be used as an argument that calcium flux is not involved in the activation of platelets by relatively impotent stimuli.

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Thrombomodulin, an Endothelial Anticoagulant Protein, Is Absent From the Human Brain

By Hidemi Ishii, Hatem H. Salem, C. Elliott Bell, Elizabeth A. Laposata, and Philip W. Majerus

Protein C activation by thrombin is significantly accelerated by the endothelial cell cofactor, thrombomodulin. In this study, we have developed a radioimmunoassay for thrombomodulin and have measured the cofactor content in several human tissues. The assay method detects as little as 2 ng of thrombomodulin. The highest thrombomodulin content was found in lung and placenta, but the antigen was also detected in spleen, pancreas, liver, kidney, skin, heart, and aorta. Unexpectedly, thrombomodulin was absent from brain. Extracts from cerebral cortex, cerebellum, centrum semiovale, midbrain, basal ganglia, pons, and medulla were devoid of thrombomodulin. In contrast, thrombomodulin antigen is present in extracerebral intracranial vessels, including basilar and internal carotid arteries and choroid plexus, as well as in endothelium of the pia-arachnoid.

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THE IMPORTANCE of protein C as a physiological anticoagulant is indicated by the finding that patients with protein C deficiency suffer thromboses.[1,2] The activated form of protein C, protein Ca, is required for anticoagulant activity. Thrombin efficiently converts protein C to Ca only when bound to a protein cofactor, thrombomodulin, that was first isolated from rabbit lung.[3] We recently have isolated the human form of thrombomodulin from placenta and have raised antibodies against this protein in rabbits.[4] We have shown that thrombomodulin antigen is distributed on the plasma membrane of human arterial, venous, capillary, and lymphatic endothelium by immunohistochemical staining of tissues.[5] In this study, we noted that thrombomodulin antigen appeared decreased or absent in vessels within the brain. We now report the development of a radioimmunoassay for thrombomodulin and the use of the assay to define the content of this moiety in several human tissues. Using the immunoassay and immunohistochemical staining of brain and related tissues, we have now established that thrombomodulin is absent from the endothelium of brain.

MATERIALS AND METHODS

Materials. Except where indicated, all reagents were from Sigma. Protein C,[6] prothrombin,[7] and antithrombin III[8] were isolated from human plasma. Human thrombomodulin was isolated from placenta as previously reported.[4] Polyclonal anti-human thrombomodulin antibody was prepared using placental thrombomodulin as the antigen.[4] Antithrombomodulin IgG was isolated from immunized rabbit serum using protein A Sepharose and was monospecific as determined by Western blots.[4] The procedures used in this study were examined and approved by the Clinical Research Committee of the Washington University School of Medicine.

Radioiodination of thrombomodulin. Carrier-free ^{125}I-sodium iodine, 100 mCi/mL, was purchased from Amersham. The iodination of thrombomodulin was performed by a modification of a technique using lactoperoxidase described by Miyachi et al.[9] Hydrogen peroxide (0.03 μmol) was added to 3 μg of human thrombomodulin in 0.1 mL of 20 mmol/L of Tris, 0.15 mol NaCl, and 0.2% Nonidet P-40(NP-40), pH 7.4, containing 500 μCi of ^{125}I-sodium iodide and 0.2 μg of lactoperoxidase. After 15 minutes, the reaction mixture was brought to 0.5 mL with the above buffer and dialyzed against the same buffer for two days. The dialyzed-labeled thrombomodulin was added to 250 μL of antithrombomodulin IgG agarose equilibrated with the above buffer and agitated for two hours at 4 °C. The agarose was washed four times with 5 mL of the same buffer. The labeled thrombomodulin was eluted with 0.5 mL of 0.2 mol/L of glycine, pH 3.0, and was immediately neutralized with 0.1 mol/L of Tris-HCl, 0.2% NP-40, pH 8.0. Antithrombomodulin IgG agarose was prepared by the method of Caldwell and Kuo.[10]

Radioimmunoassay. Reaction mixtures contained the following: test sample, 0.25 μg of antithrombomodulin IgG (an amount that gives 30%–90% precipitation of ^{125}I-thrombomodulin tracer) in 5 μL and 0 5 μL of nonimmune rabbit serum in 20 mmol/L of Tris-HCl, pH 7 4, 0.15 mol/L of NaCl, and 0.2% NP-40, in 0.2 mL. After incubation at 4 °C in 1.5-mL Eppendorf tubes (Brinkman) for two hours, ^{125}I-thrombomodulin (16,000 cpm, 8,000 cpm/ng) was added, and the incubation was continued for an additional 16 hours. Eighty microliters of 10% suspension of *S aureus* (Pansorbin, Calbiochem, La Jolla, Calif) was then added to each reaction mixture, and incubation was continued for an additional hour at room temperature. The *S aureus* organisms were collected by centrifugation at 14,000 g for ten minutes, the supernatant solutions were removed, and the ^{125}I-thrombomodulin bound was measured in a gamma scintillation spectrometer.

Tissues were obtained from autopsies performed within six hours after death and were kept frozen at −20 °C. The frozen tissues were shredded using a vegetable shredder and were homogenized in 20 mmol/L of Tris, pH 7.4, 0.15 mol/L of NaCl, and 0.2% NP-40 in a Polytron tissue homogenizer (Brinkmann Inst. Co., Westbury, NY). The thrombomodulin in homogenates was solubilized by stirring at 4 °C for four hours. Platelets, erythrocytes, lymphocytes, and granulocytes were isolated as described elsewhere.[11]

Detection of thrombomodulin antigen by an immunohistochemical method The immunohistochemical staining of thrombomodulin antigen was carried out by an avidin–biotin immunoperoxidase method as previously reported.[5]

RESULTS

Thrombomodulin content in human tissues. We previously showed that our rabbit antithrombomodulin IgG is monospecific for human thrombomodulin by immunoblotting.[4] In addition, we found that the antibody inactivates the protein C activating cofactor activity of thrombomodulin. One microgram of the IgG inactivates ~13 ng of thrombomodulin.[12] In preliminary experiments, we found that only

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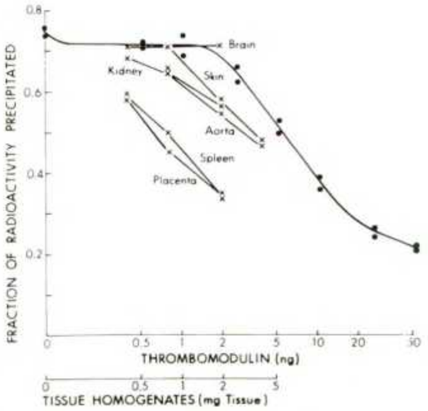

Fig 1. Radioimmunoassay of thrombomodulin purified from placenta (●) and in extracts of various tissues (x).

Table 1. Distribution of Thrombomodulin in Several Human Tissues

	Exp. 1	Exp. 2	Exp. 3 (μg/g Tissue)	Exp. 4	Mean ± SD
Lung	11.9	6.2	—	9.8	9.3 ± 3.0
Heart	2.7	3.7	—	—	3.2
Liver	4.5	6.9	—	1.5	4.3 ± 2.2
Spleen	5.6	8.9	7.4	3.4	6.3 ± 2.1
Kidney	2.7	4.0	2.4	2.8	3.0 ± 0.6
Pancreas	1.6	3.8	—	11.3	5.6 ± 5.1
Aorta	0.8	2.8	2.4	2.2	2.1 ± 0.8
Skin	—	1.0	1.9	2.2	1.7 ± 0.5
Placenta	8.2	9.9	8.0	6.9	8.3 ± 1.1

The tissues in experiment 1 were obtained from a 79-year-old man who died from Alzheimer's disease. The tissues in experiments 2 and 3 were obtained from men, 36 and 25 years old, respectively, who died in accidents. The tissues in experiment 4 were obtained from a woman, 30 years old, who died from alcoholic hepatitis. The values for placenta were obtained from normal-term placentas.

Table 2. Distribution of Thrombomodulin in Several Areas of Brain and ExtraCerebral Vessels

	Number of Brains Studied	Thrombomodulin Content (μg/g Tissue)
Cerebral cortex	6	0
Cerebellum	3	0
Basilar artery	1	0.8
Internal carotid artery	1	1.4
Centrum semiovale	2	0
Choroid plexus	2	1.45
Midbrain	1	0
Basal ganglia	1	0
Pons	1	0
Medulla	1	0

Brain tissues were obtained from men who died in accidents.

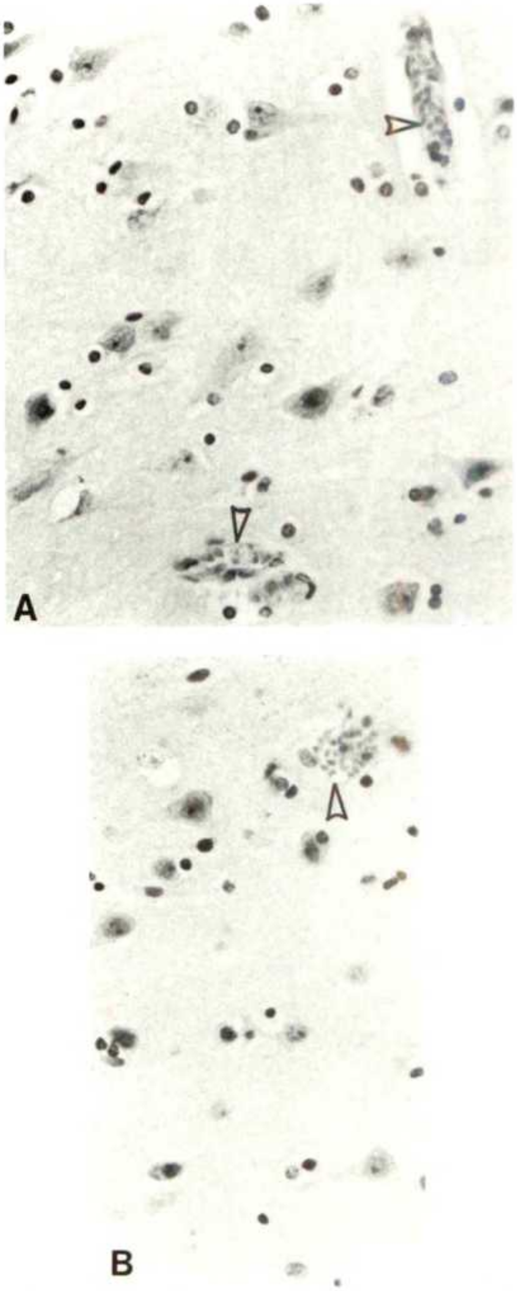

Fig 2. Immunohistochemical staining for thrombomodulin in gray matter of occipital cortex. Rabbit antithrombomodulin IgG failed to stain blood vessel endothelial cells (open arrowheads) in different areas (A and B) by an avidin–biotin immunoperoxidase method. Red blood cells exhibit faint endogenous peroxidase staining. Hematoxylin counterstain. Original magnification ×500; current magnification ×385.

the lactoperoxidase method of iodination was satisfactory to prepare thrombomodulin that was stable and that retained full biological activity. Iodination with iodogen,[13] Bolton-Hunter reagent,[14] and Chloramine T[15] all proved unsatisfactory.

Radioimmunoassay of thrombomodulin was performed as described in the Methods section, and a typical standard

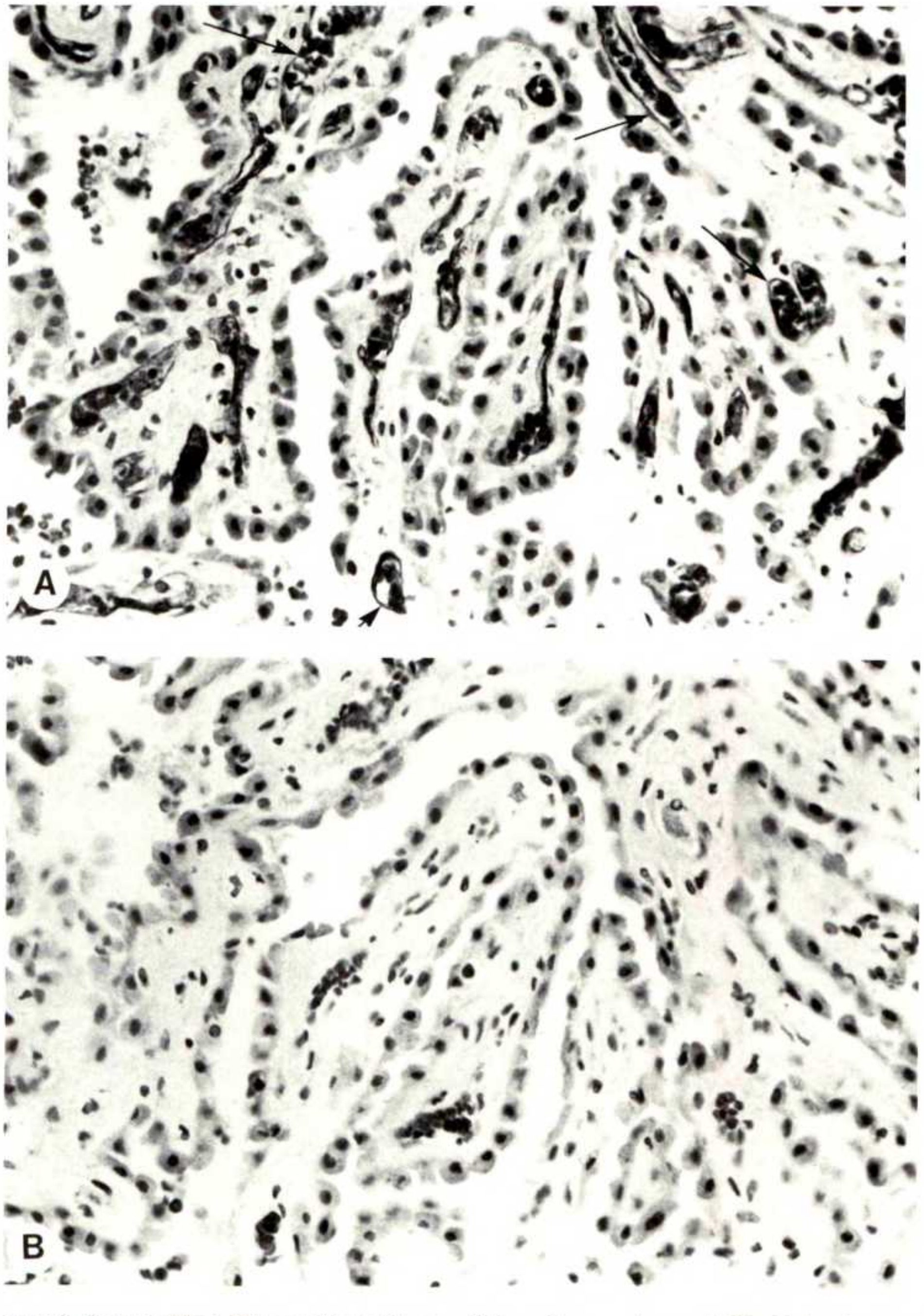

Fig 3. Immunohistochemical staining for thrombomodulin in choroid plexus. Rabbit antithrombomodulin IgG (A) stained capillary endothelial cells (solid arrowheads), whereas rabbit control IgG (B) failed to give staining. In A, no other cells gave positive immunoperoxidase staining, including the cuboidal epithelial cells on the surface of the plexuses. Hematoxylin counterstain. Original magnification ×500; current magnification ×385.

curve is shown in Fig 1. We are able to detect as little as 2 ng of thrombomodulin with 50% displacement of ^{125}I-tracer by 5 to 10 ng of unlabeled thrombomodulin in various experiments.

In preliminary experiments, we showed that the method used to solubilize thrombomodulin from placenta provides a suitable soluble extract for immunoassay. Repeated extraction of the insoluble residue of placenta did not yield additional antigen. We used the same method to prepare extracts of other organs. The thrombomodulin content of several tissues is shown in Table 1. The highest content of thrombomodulin was found in lung and placenta. We are unable to account for the high values obtained for pancreas in case 4. The high value was confirmed by a functional assay for protein C activating cofactor activity, and the pancreas in this case was histologically normal. None of the organ extracts contained materials that interfere with the assay, since we added isolated placental thrombomodulin to extracts of each tissue and found complete recovery by immunoassay. The slopes of the assays for placenta, spleen, kidney, skin, and aorta were similar to the standard curve as shown in Fig 1, further substantiating that thrombomodulin is accurately measured. We assayed for thrombomodulin in circulating blood cells including platelets, erythrocytes, lymphocytes, and granulocytes, and none was found.

Thrombomodulin in brain. We were unable to detect thrombomodulin antigen in brain, as shown in Table 2 and Fig 1. Thrombomodulin antigen was found in intracranial vessels including basilar artery, internal carotid artery, and choroid plexus (Table 2). However, the endothelium of vessels within the substance of the brain apparently lacks thrombomodulin antigen. We assayed numerous samples from different areas of brain, and no antigen was found. The lack of thrombomodulin antigen was not due to the presence of an inhibitor in brain, since brain extracts did not inhibit immunoassay of thrombomodulin when mixed with either purified thrombomodulin or extracts of other tissues. Furthermore, we could not detect any functional thrombomodulin in brain measured as protein C activating cofactor. We further examined sections of brain by the immunohistochemical method shown in Figs 2 and 3. Neither the rabbit antithrombomodulin IgG (Fig 2) nor the rabbit control IgG (not shown) reacted with endothelial cells in cerebral cortex. In contrast, thrombomodulin was easily detected in choroid plexus by this method as shown in Fig 3. Rabbit antithrombomodulin IgG, but not control IgG, also stained endothelial cells in capillaries of the pia-arachnoid (not shown).

DISCUSSION

Thrombomodulin is widely distributed in the endothelium of arteries, veins, capillaries, and lymphatics.[5] We used immunoassay of extracts of various tissues to estimate the content of this antigen in various organs. The highest content of thrombomodulin was found in lung and placenta. There are few antigens described that are unique to endothelium. von Willebrand factor is a standard antigen used to define endothelium, although it is also contained in placental syncytiotrophoblasts,[5] megakaryocytes,[16] and platelets.[17] Thrombomodulin is found only on endothelium and placental syncytiotrophoblast cells. Brain endothelium is functionally different from other endothelia in the body since the blood-brain barrier prevents free access of materials in plasma to the extracellular fluid of brain.[18] In addition to the presence of tight junctions[19,20] and lack of vesicles, a number of enzymes, including alkaline phosphatase, γ-glutamyl transpeptidase and Na^+,K^+-ATPase are present in higher concentration in cerebral endothelium than in other tissues.[18] The lack of thrombomodulin in cerebral endothelium is another difference between brain and other endothelia. The basis for this difference is unknown. Thrombomodulin was not detected in white matter and gray matter such as cerebral cortex, cerebellum, midbrain, centrum semiovale, basal ganglia, pons, and medulla in brain. The physiological significance of lack of thrombomodulin in white and gray matter in brain is uncertain. Perhaps the possibility of intracerebral hemorrhage is so threatening to survival that the molecule is not contained in vessels that are within the substance of the brain. However, basilar and internal carotid arteries, pia-arachnoid, and choroid plexus which supply the brain, do contain thrombomodulin.

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Synergistic Effects of Butyrate on Platelet Responses to Arachidonate, A23187, PGE$_1$, and Forskolin

By Narendra N. Tandon, Tommie S. Tralka, and G.A. Jamieson

With eukaryotic cells, butyrate is known to induce a series of morphological and biochemical changes that mimic cellular differentiation. With platelets, we have found that butyrate (10 mmol/L) caused an approximately threefold increase in sensitivity to calcium ionophore A23187 and arachidonate. Maximum aggregation was observed at agonist concentrations of 3 μmol/L and 170 μmol/L, respectively, as compared with required concentrations of 10 μmol/L and 400 μmol/L in the absence of butyrate. Similar effects were seen with isobutyric acid, and about one-half the effect was shown with valerate and caproate, but lower homologues showed no synergistic effect. No ultrastructural changes were observed in platelets incubated with butyrate, and the aggregation effects were reversible and returned to normal on removal of butyrate. Membrane fluidity was unchanged by butyrate as measured by changes in the fluorescence depolarization of diphenylhexatriene. Butyrate caused a 60% to 70% increase in the uptake of ^{3}H-arachidonate. Butyrate also potentiated the inhibition of platelet function by prostaglandin E$_1$ and forskolin and uptake of ^{3}H-forskolin was increased ~20%. In contrast, platelet response to other agonists (ADP, epinephrine, collagen, thrombin, and platelet-activating factor) was essentially unaffected by butyrate. These results suggest that butyrate may increase the uptake of certain hydophobic agonists and antagonists by platelets. Similar mechanisms for uptake of endogenous effectors may explain the response of eukaryotic cells to butyrate in culture.

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THE EFFECTS OF sodium butyrate on eukaryotic cells in culture have been extensively studied since this agent appears able to induce a wide variety of biochemical and ultrastructural changes which mimic the changes associated with cellular differentiation (for review, see reference 1). In eukaryotic cells, the effects of butyrate include changes in the cell cycle[2,3] and the expression of tissue factor activity[4] and enhanced adhesion of cells to glass with concomitant elongation and flattening,[5–7] as well as induction of β-adrenergic receptors[8] and of membrane-associated activities such as glycolipid biosynthesis[9,10] and sialyl transferase activity.[11] At the nuclear level, butyrate affects chromatin,[12–14] vimentin filaments,[15] and the hyperacetylation of histones H3 and H4 in eukaryotic cells.[16–21]

The mechanism of butyrate effects on eukaryotic cells is not known. The hyperacetylation of histones is thought to play a key role in transcription of RNA, both qualitatively and quantitatively, thus suggesting one possible mode of butyrate action. However, there is recent evidence which does not support this hypothesis. First, it has been shown that hyperacetylation of histones is evident in both transcribed and nontranscribed chromatin.[22] Second, in the *Physarum* cell cycle, histone H4 acetylation and transcriptional activity are uncorrelated.[23] Furthermore, Rubenstein et al[24] have shown cellular protein synthesis and inhibition of cell division to be independent of hyperacetylation effects. Thus, this postulated mechanism of butyrate action on eukaryotic cells is still not confirmed.

Platelets are cell fragments which lack a nucleus and have little or no ability for protein synthesis. Because they lack transcriptional and translational activity and because of the importance of membrane effects in their function, platelets appeared to provide a suitable model system in which to study the effect of butyrate in the absence of nucleic acid changes and related eukaryotic cell functions. The results presented here suggest that butyrate can profoundly affect platelet function, and that it may exert its effects by increasing the uptake of hydrophobic agonists and antagonists into platelets. These results may have bearing on the role of butyrate in the apparent transformation of eukaryotic cells.

MATERIALS AND METHODS

The following compounds were purchased from Sigma Chemical Co, St Louis: L-epinephrine, imipramine, adenosine diphosphate (ADP), bovine serum albumin (BSA) (essentially free of fatty acids), arachidonic acid, bovine Achilles tendon collagen, butyric acid, isobutyric acid, β-hydroxy butyrate (Na$^+$-salt), Na-formate, acetic acid, propionic acid, valeric acid, and caproic acid. Prostaglandin E$_1$ (PGE$_1$), was obtained from Upjohn Co, Kalamazoo, Mich. Stock solutions (2.8 μmol/L) were prepared in ethanol, stored at −20 °C, and diluted in Tyrode's buffer just before use. Calcium ionophore A23187, platelet-activating factor and forskolin were obtained from Calbiochem-Behring Corp, La Jolla, Calif. Forskolin was dissolved in ethanol and stored at −20 °C Compound A23187 was stored at −20 °C as a 10 mmol/L solution in dimethyl sulfoxide. Arachidonic acid was dissolved in ethanol at a final concentration of 150 mmol/L and stored in small aliquots at −20 °C in sealed tubes flushed with nitrogen. At the time of use, stock solutions of A23187, forskolin, and arachidonic acid were diluted with ethanol as needed· when added to the platelet suspension, the final concentration of ethanol did not exceed 0.5%. ^{14}C-Serotonin (5-hydroxytryptamine creatinine sulfate; 58 Ci/mol), and U^{14}C-sucrose (10 mCi/mmol) were obtained from Amersham/Searle Corp, Arlington Heights, Ill, 5,6,8,9,11,12,14,15-^{3}H Arachidonic acid (60 to 100 Ci/mmol), 1-^{14}C-butyric acid Na-salt (13.4 mCi/mmol), Protosol, 12-^{3}H-forskolin-(31 Ci/mmol) and ^{45}Ca^{2+} (4 to 50 Ci/g calcium) were purchased from New England Nuclear Corp, Boston. Purified human fibrinogen was from Kabi, Stockholm, Sweden. Hiphenyl silicone 125 DC-550 and methyl-silicone 2.0 DC-220 were obtained from William F. Nye, Inc, New Bedford, Mass. All other chemicals used were of reagent grade.

Platelet preparations. Fresh human blood anticoagulated with citrate-phosphate-dextrose-adenine was obtained from the Wash-

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ington Region, American Red Cross Blood Services. Platelet-rich plasma was obtained from whole blood by centrifugation at 3,400 rpm (3,200 g) for 3.5 minutes at room temperature in a Sorvall centrifuge (Model RC-3). To ensure removal of contaminating red cells and leukocytes, platelet-rich plasma was further centrifuged at 180 g for ten minutes at room temperature.

For the preparation of washed platelets, the pH of the platelet-rich plasma was lowered to 6.5 with citric acid (1 mol/L), and platelets were sedimented by centrifugation at 1,800 g for ten minutes. Platelet wash buffer (pH 6.5; 5.5 mmol/L of dextrose, 128 mmol/L of NaCl, 4.26 mmol/L of NaH_2PO_4, 7.46 mmol/L of Na_2HPO_4, 4.77 mmol/L of trisodium citrate, 2 35 mmol/L of citric acid and 0.3 g/dL of BSA) was added without disturbing the platelet pellet. After 15 to 20 minutes at room temperature, the pellet was suspended in the supernatant buffer, and the suspension was then centrifuged at 800 g for four minutes (Serofuge, Clay-Adams, Inc, New York).

To prepare a platelet suspension for aggregation studies, the twice-washed platelet pellet was suspended in modified Hepes-Tyrode's buffer, pH 7.35 (136 mmol/L of NaCl, 5.5 mmol/L of dextrose, 1 mmol/L of $MgCl_2$, 0.47 mmol/L NaH_2PO_4, 11.62 mmol/L of $NaHCO_3$, 2.7 mmol/L of KCl, 10 mmol/L of Hepes, and 0.3 g/dL of BSA) and was diluted to a final concentration of about 3 to 3.5 × 10^8 cells per milliliter in a solution made 1 mmol/L with respect to Ca^{2+} and 1 mg/mL with respect to human fibrinogen. Platelets were counted in a Coulter Counter (Coulter Electronics, Hialeah, Fla).

Platelet incubations. Platelets in plasma or washed platelets in Hepes-Tyrode's buffer were incubated with and without sodium butyrate (10 mmol/L, pH 7.4) for 1 hour at 37 °C at a platelet concentration of 3 × 10^8 per milliliter and then kept at room temperature until studied. When secretion was to be studied, platelet-rich plasma was incubated for 45 to 60 minutes at 37 °C with ^{14}C-serotonin (1 μmol/L final concentration), by which time 90% to 96% of ^{14}C-serotonin had been taken up by the platelets. The labeled platelet suspension was then held at room temperature until used.

Separation of radiolabeled platelets. In several series of experiments, platelets were incubated with radioactive agents (arachidonate, forskolin, Ca^{2+}, etc), and it was necessary to separate the labeled platelets from the incubation mixture. For this purpose, samples (100 μL) were withdrawn, layered onto a 25-μL volume of silicone oil (Hiphenyl: methylsilicone, 4:1 vol/vol) in a polypropylene conical centrifuge tube, and centrifuged at 12,000 g for 30 seconds to sediment the platelets. The oil and the supernatant fluid were removed by suction, and the bottom of the tube containing the platelet pellet was cut off and transferred to a scintillation vial containing protein solubilizer (Protosol). After overnight incubation at room temperature, 40 μL of glacial acetic acid/0.5 mL Protosol and 10 mL of scintillation fluid were added, and radioactivity was counted.

Aggregation studies. Aggregation studies were performed turbidimetrically in a Payton Aggregometer (Payton Associates, Buffalo). Cuvettes (1.5 mL), containing 0.45 to 0.5 mL of platelet suspension were stirred at 950 rpm at 37 °C for one minute prior to the addition of 1 to 10 μL of the various aggregating agents. In studies on the release of ^{14}C-serotonin, reuptake was blocked by the addition of 1 to 2 μmol/L of imiprimine one to two minutes prior to the addition of platelet agonists. Three minutes after the addition of the aggregating agent, the reaction was terminated by the addition of one-tenth vol of a mixture of formaldehyde (3%) and EGTA (50 mmol/L) The platelet suspension was centrifuged at 8,000 g for two minutes and released ^{14}C-serotonin was counted in the supernatant solution.

$^{45}Ca^{2+}$ uptake by platelets. Washed platelets (1 × 10^9 per milliliter) in Hepes-Tyrode's buffer were incubated for one hour with and without sodium butyrate (10 mmol/L) at 37 °C. The reaction was initiated by the addition of Ca^{2+} (10 μCi $^{45}Ca^{2+}$/mL with unlabeled $CaCl_2$, final concentration 0.5 mmol/L). The contents were mixed by gentle inversion and then incubated at 37 °C Platelets were separated from the incubation mixture as described above, and radioactivity was determined. To determine trapped volume, $^{45}Ca^{2+}$ and 8 mmol/L of EGTA were incubated together for five minutes prior to addition of the platelet suspension. Platelets were then centrifuged through silicone oil as described above and remaining radioactivity was determined.

^{14}C-Butyrate influx and efflux Washed platelets (1 × 10^9/mL) in Hepes-Tyrode's buffer were incubated with ^{14}C-butyrate (1 μCi/mL; total butyrate 75.0 μmol/L) at 37 °C. Samples (100 μL) were withdrawn at intervals from 0 to 90 minutes, and the radioactivity of the platelets was counted after centrifugation through oil, as described above. For the efflux study, 1 vol of the butyrate-labeled platelet suspension (at 90 minutes) was diluted with 2 vol of platelet wash buffer, centrifuged at 900 g for four minutes, and resuspended in the original volume of Hepes-Tyrode's buffer. The platelet suspension was returned to 37 °C, 100-μL samples were removed and centrifuged through silicone oil at various times, and radioactivity was determined in the platelet pellet as described above. The entrapped volume of the platelet pellet in this and other uptake experiments was determined as described by Brass and Shattil,[25] but using ^{14}C-sucrose.

^3H-Arachidonic acid uptake and mobilization. To measure arachidonic acid uptake, washed platelets (2 × 10^9 per milliliter) in Hepes-Tyrode's buffer were incubated with and without butyrate (10 mmol/L) for 45 minutes at 37 °C; aliquots (250 μL) of the suspensions were then removed, and the reaction was started by adding equal volumes of Hepes-Tyrode's buffer with differing concentrations of arachidonate (final concentration 10 to 100 μmol/L) each containing 0.5 μCi of ^3H-arachidonate To avoid platelet aggregation, the contents of the tubes were mixed by gentle inversion and were then incubated for three minutes at 25 °C. Aliquots (100 μL) were withdrawn, the platelets were recovered by centrifugation through oil, and their radioactivity was determined as described above.

When agonist-induced arachidonic acid mobilization was to be studied, washed platelets (4 × 10^8 per milliliter) were incubated with and without butyrate for 30 minutes at 37 °C These platelet suspensions were then further incubated with ^3H-arachidonic acid (1 μCi/mL, 7.4 μmol/L) for another 30 minutes at 37 °C. At the end of the incubation, the platelets were washed once with platelet wash buffer and resuspended into the original volume of Hepes-Tyrode's buffer. Except when response to A23187 was to be measured, the platelet suspension had 1 mmol/L of Ca^{2+} and 1 mg/mL of fibrinogen. Three minutes after the addition of agonists, the reaction was stopped by addition of chilled EGTA, and the platelet suspension was centrifuged at 8,000 g for two minutes The radioactivity in the supernatant solution was determined using an Intertechnique liquid scintillation counter (Model SL 4000) at an efficiency of 40% for ^3H and 90% for ^{14}C. Platelet cyclic AMP was measured by a radioimmunoassay kit (New England Nuclear Corp, Boston) according to Steiner et al.[26]

^3H-Forskolin incorporation. To measure the uptake of forskolin, washed platelets (2 × 10^9 per milliliter) in Hepes-Tyrode's buffer (pH 7.4) without albumin were incubated with and without butyrate (10 mmol/L) for 45 minutes at 37 °C and then at room temperature for 15 minutes. Aliquots (250 μL) were removed and maintained on melting ice, and the reaction was started by immediately adding equal volumes of chilled Hepes-Tyrode's buffer (without albumin), with differing concentrations of forskolin (final concentration 0 to 0.6 μmol/L) each containing ^3H-forskolin (0.5 μCi). The reaction was vortexed briefly to ensure complete mixing, and samples were

processed at appropriate times for incorporated radioactivity as described above.

Fluorescence polarization Changes in membrane microviscosity were measured as changes in fluorescence polarization using platelets labeled with diphenylhexatriene (DPH) as described earlier.[27]

RESULTS

Aggregation. In preliminary studies, it was found that butyrate effects could be observed only when platelets, whether washed or in plasma, had been incubated with butyrate for 30 to 60 minutes at 37 °C. In the experiments described, platelets were incubated with butyrate for one hour at 37 °C unless otherwise indicated. The buffered butyrate solutions did not affect the pH of the reaction mixtures.

When low concentrations of ionophore A23187 (3 μmol/L) were added to platelet-rich plasma, only a reversible first wave of aggregation occurred (Fig 1A, curve 1). However, when the ionophore was added following incubation with butyrate, full irreversible aggregation was obtained (Fig 1A, curve 2). In the absence of butyrate, full aggregation required ionophore concentrations of 8 to 10 μmol/L. Butyrate itself had no effect on light transmission of washed platelet preparations or platelet-rich plasma in the aggregometer.

Similar results were observed with arachidonic acid (166 μmol/L). This concentration of arachidonate induced a slight reversible aggregation of platelet-rich plasma (Fig 1B, curve 1) whereas, if added after incubation with butyrate, full irreversible aggregation was achieved (Fig 1B, curve 2). In the absence of butyrate, arachidonate concentrations of 300 to 400 μmol/L were required for full aggregation.

These results were obtained with butyrate concentrations of 10 mmol/L, but similar results were obtained at concentrations of 5 mmol/L. However, decreases of butyrate below this concentration resulted in complete loss of the synergistic effect, and the aggregation patterns were essentially identical with those of controls when platelets were challenged with arachidonate or ionophore A23187. Synergistic effects were not seen with low concentrations of ADP, collagen, thrombin, epinephrine, and platelet-activating factor. A slight inhibition of aggregation (~10%) was occasionally observed with some platelet preparations but was not investigated further.

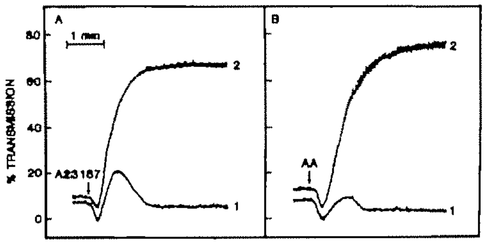

Fig 1. Effect of butyrate on platelet aggregation. Platelets (2.5 to 3.5 × 10^8/mL) in plasma were incubated with and without butyrate (10 mmol/L) for 45 to 60 minutes at 37 °C: (A) Ionophore (A23187)-induced aggregation. Curve 1, A23187 alone (3 μmol/L); curve 2, A23187 (3 μmol/L) plus butyrate. (B) Arachidonic acid-induced aggregation. Curve 1, arachidonate alone (166 μmol/L); curve 2, arachidonate (166 μmol/L) plus butyrate.

Of the isomers of butyric acid tested, isobutyric acid showed similar effects on platelet aggregation with ionophore A23187 and arachidonate as did butyrate itself. However, β-hydroxybutyrate had no effect on platelet response at 10 mmol/L concentration. At this concentration, the butyrates had no effect on basal levels of cyclic AMP or the levels observed following stimulation with PGE_1 (data not shown). Higher and lower homologues of butyric acid were also tested. Formate, acetate, and propionate had no effect, whereas the next higher homologues, valerate (C_5), and caproate (C_6), at 10 mmol/L concentration gave about one-half of the enhancing effect seen with butyrate.

Paper chromatographic analysis of radioactivity after two hours of incubation of platelets with ^{14}C-butyrate, in the solvent system acetone: *t*-butanol: *n*-butanol: NH_4OH::2:1:1:1, revealed that all the radioactivity corresponded to ^{14}C-butyrate, indicating that platelets do not metabolize butyrate during this period.

Fluorescence polarization. The value of fluorescence polarization at 27 °C (P_{27}) of diphenylhexatriene incorporated into control platelets was 0.275 ± 0.018 (n = 5). Following 45 minutes of incubation of platelets with butyrate (10 mmol/L), which resulted in enhanced aggregation by A23187 and arachidonate, there was no change in the fluorescence polarization value (P_{27}, 0.273 ± 0.012; n = 3).

Serotonin release. To determine whether butyrate affected only platelet aggregation or both aggregation and secretion, platelets in plasma were labeled with ^{14}C-serotonin, and the extent of aggregation and release were measured during aggregation induced by ionophore, both in the presence and absence of added butyrate (Table 1).

The amount of serotonin incorporated into platelets was similar both in the presence and absence of butyrate (~ 95%). When added to platelet suspensions in the absence of agonists, butyrate did not induce any release of ^{14}C-serotonin. When the concentration of ionophore was reduced from 10 μmol/L in a stepwise fashion, full platelet aggregation was observed in the butyrate-treated sample at concentrations as low as 3 μmol/L, by which time aggregation in the control sample had fallen to 56%. Further reduction to 1 μmol/L resulted in reversible aggregation in the control, which reached a value of 32% before disaggregation, whereas the butyrate-treated sample continued to show irreversible aggregation to a level of 54% (data not shown).

Similar trends were seen with serotonin release at ionophore concentrations of 3 μmol/L: release in the control sample was only 6% of the total taken up, whereas release in the butyrate-treated sample was 20%, or more than threefold greater.

Trapped volume. Trapped volume in platelet pellets following centrifugation through silicone oil was determined in studies of uptake and efflux of various agents such as butyrate, arachidonate, Ca^{2+}, and forskolin. This was determined in two ways: for studies on Ca^{2+} flux in unstimulated platelets, trapped volume was determined by addition of EGTA and was equal to 0.22 ± 0.3 μL/10^8 platelets, corresponding to ~0.2% of the added radioactivity. In the

Table 1. Effect of Butyrate on Platelet Aggregation and Serotonin Release

	Serotonin Release (%)		Aggregation (%)	
	Butyrate		Butyrate	
A23187 (μmol/L)	(−)	(+)	(−)	(+)
10	20	31	75	75
5	13	29	66	75
3	6	21	56	74

Platelet-rich plasma was used.

studies with butyrate, arachidonate, and forskolin, trapped volume using ^{14}C-sucrose was found to be 0.31 ± 0.09 μL/10^8 platelets, corresponding to 0.3% of the added radioactivity, a value similar to that found with EGTA. Trapped volume was not significantly affected by the presence of aggregates induced by arachidonate. All values were corrected for trapped volume.

Influx and efflux of ^{14}C-butyrate. On incubation at 37 °C, ^{14}C-butyate was incorporated into washed platelets in a linear fashion up to 45 minutes, but uptake then plateaued, with maximal incorporation of 0.92 nmol ^{14}C-butyrate/10^8 platelets (Fig 2). If platelets were resuspended in butyrate-free medium, there was a rapid fall in intracellular ^{14}C-butyrate concentration up to 30 minutes with a slower rate of efflux up to 90 minutes (Fig 3). When platelets were first loaded with butyrate (10 mmol/L for one hour at 37 °C) and were then transferred to butyrate-free medium and further incubated for one hour at 37 °C, no difference in aggregation patterns was observed between control and butyrate-depleted platelets (data not shown).

Uptake and mobilization of arachidonate. When ^3H-arachidonate was added to washed platelet suspensions, uptake reached a maximum at 15 minutes both in the control and butyrate-treated samples. Studies of the concentration dependence of arachidonate incorporation showed that arachidonate uptake was increased ~70% in the presence of butyrate as compared with control samples (Fig 3). In separate experiments, these arachidonate-labeled platelets were examined to determine the degree of mobilization of arachidonate in response to ionophore (Table 2). There appeared to be a strong parallel between the effect of butyrate on aggregation and the degree of mobilization of ^3H-arachidonate. The lowest concentrations of ionophore (0.25 μmol/L) gave maximum aggregation and arachidonate mobilization in the presence of butyrate, whereas there was virtually no observable aggregation (10%) or mobilization (0.4%) in its absence.

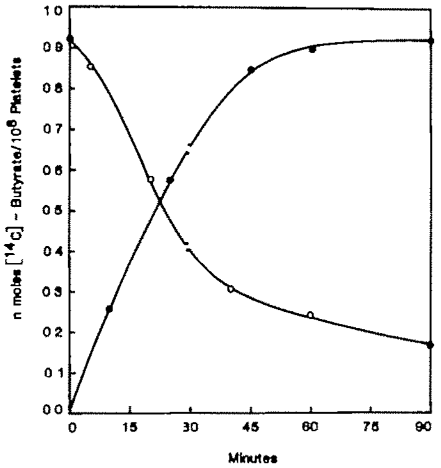

Fig 2. Transport of butyrate into washed platelets. Washed platelets (5 × 10^8/mL) were incubated with ^{14}C-butyrate, and the influx into the platelets was measured (—●—). Once equilibrium was reached, the platelets were transferred to buffer, and the rate of efflux was measured (—○—).

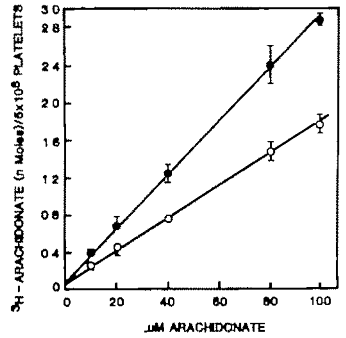

Fig 3. Uptake of arachidonic acid by washed platelets in the absence of 10 mmol/L of butyrate (—○—) and in its presence (—●—). Data are mean ± SD of two experiments run in triplicate.

$^{45}Ca^{2+}$ Uptake. The uptake curve for $^{45}Ca^{2+}$ was essentially identical in the presence and absence of butyrate, indicating that butyrate as such did not affect direct uptake of Ca^{2+} by unstimulated platelets (Fig 4).

Effects of PGE_1 and forskolin. The inhibitory effect of forskolin and PGE_1 on arachidonic acid-induced platelet aggregation was studied in the presence and absence of added butyrate (Figs 5A and B). Addition of PGE_1 (1.5 nmol/L) to the control washed platelet suspension inhibited the arachidonic acid-induced aggregation by 60% (Fig 5A, curve 3), whereas in butyrate-treated platelets, the same concentration of PGE_1 resulted in complete inhibition of aggregation induced by arachidonic acid (Fig 5A, curve 4). Similarly, forskolin (0.15 μmol/L) inhibited arachidonic acid-induced aggregation by 40% in the control group (Fig 5B, curve 3), whereas in the butyrate-treated group, the same low concentration of forskolin resulted in a complete inhibition of platelet aggregation (Fig 5B, curve 4).

Uptake of ^3H-forskolin. Studies of the time dependence of ^3H-forskolin uptake into platelets at 37 °C showed that

Table 2. Effect of Butyrate on Aggregation and ^3H-Arachidonic Acid Mobilization Induced by Ionophore A23187

	Aggregation (%)		Arachidonate Mobilization (%)	
	Butyrate		Butyrate	
A23187 (μmol/L)	(−)	(+)	(−)	(+)
2.5	80	80	25	26
1.0	75	80	11	30
0.5	70	80	4	11
0.25	10	80	0.4	4

Washed platelets were used.

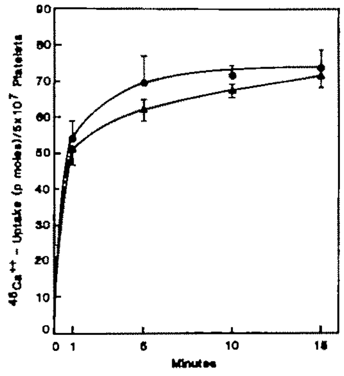

Fig 4. Uptake of Ca^{2+} by washed, unstimulated platelets in the presence (- -●- -) and absence (- -▲- -) of butyrate (10 mmol/L). Data are mean ± SD of two experiments run in triplicate.

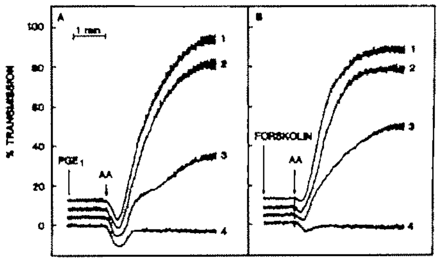

Fig 5. Effect of butyrate (10 mmol/L) on inhibition by PGE_1 and forskolin of platelet aggregation induced by arachidonate. (A) Inhibition by PGE_1 (1.5 nmol/L). Curve 1, arachidonate (18.75 μmol/L) in the presence of butyrate; curve 2, arachidonate alone (45 μmol/L); curve 3, arachidonate (45 μmol/L) plus PGE_1 (1.5 nmol/L); curve 4, arachidonate (18.75 μmol/L) plus PGE_1 (1.5 nmol/L) in the presence of butyrate. (B) Inhibition by forskolin (0.15 μmol/L). Curve 1, arachidonate (30 μmol/L) in the presence of butyrate; curve 2, arachidonate alone (60 μmol/L); curve 3, arachidonate (60 μmol/L) plus forskolin (0.15 μmol/L); curve 4, arachidonate (30 μmol/L) plus forskolin (0.15 μmol/L) in the presence of butyrate.

maximum values were reached even at the shortest time point that could be measured (~15 seconds), and that uptake in the presence of butyrate was 10% higher than in control samples for the first minute but declined thereafter so that uptake was equal in both control and butyrate-treated groups after two minutes (Fig 6A). Because these changes could have been due to metabolism of forskolin or other effects, we examined the uptake of forskolin in platelets that had been briefly chilled to 0° to 10°. When the time-dependence was measured at 0° to 1°, maximal uptake was again observed by 15 seconds; however, there was no subsequent decrease, and the uptake into samples incubated in the presence of butyrate remained about 20% higher than in control samples up to four minutes, the maximum time point measured (Fig 6B). Studies of the concentration dependence of ^3H-forskolin uptake showed that this difference was maintained at least up to 0.6 μmol/L (Fig 6C).

DISCUSSION

Previous studies in relation to the interaction of platelets with dibutyryl cyclic 3,′5′-guanosine monophosphate showed that low concentrations of butyrate (10 μmol/L) had no effect on platelet function.[28] In contrast, the present studies show that higher concentrations of butyrate (5 to 10 mmol/L) result in an approximately three-fold increase in the sensitivity of platelets to the stimulatory effects of arachidonate and A23187 and to the inhibitory effects of PGE_1 and forskolin: that is, the effects of these reagents, whether stimulatory or inhibitory, were enhanced by these concentrations of butyrate. The basis of these effects in the presence of butyrate would appear to be the increased uptake of the agonists and antagonists into platelets: these increases were about 60% for arachidonate and about 20% for forskolin at all the concentrations studied. The reason for this increased uptake in the presence of butyrate is not known but could arise from changes in the solution-phase state of the reagents or in their partitioning into and across the platelet membrane.

The agents which show these effects are either hydrophobic neutral molecules (forskolin, ionophore, and PGE_1) or, as in the case of arachidonate, contain a significant proportion of hydrophobic neutral species at physiological pH. In contrast, the platelet-aggregating agents which do not show a butyrate effect (ADP, thrombin, collagen, epinephrine, and platelet-activating factor) all contain ionic residues under these conditions. These results could indicate that butyrate has a general detergent effect on cell membranes as previously suggested.[1] Although the absence of butyrate effects on fluorescence polarization of DPH suggests no change in bulk membrane fluidity, butyrate could be exerting an effect on membrane microdomains not detectable by this technique.

Ca^{2+} homeostasis is important in maintaining platelets in the unstimulated form.[29] The presence of butyrate did not lead to any significant change in Ca^{2+} flux into unstimulated

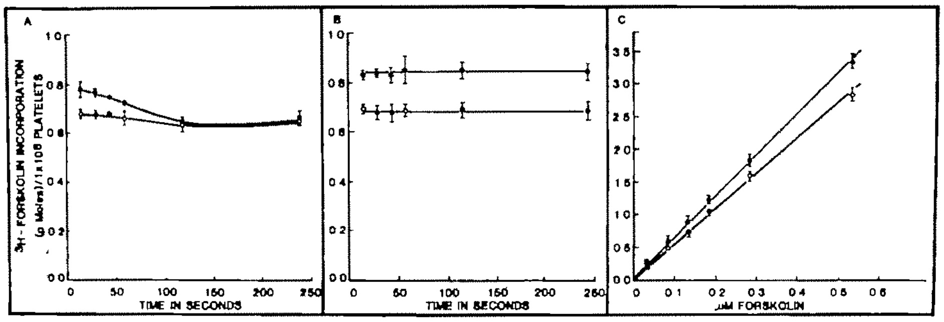

Fig 6. Time and concentration dependence of forskolin uptake by platelets. Filled symbols, uptake of forskolin in the presence of added butyrate (10 mmol/L); open symbols, uptake of forskolin in the absence of added butyrate. (A) Uptake of forskolin (0.16 μmol/L) at 37 °C. (B) Uptake of forskolin (0.16 μmol/L) at 0° to 1°. (C) Forskolin (0 to 0.6 μmol/L) uptake at 0° to 1°.

platelets. Although Ca^{2+} flux into platelets was approximately doubled following stimulation with arachidonate or A23187 in the presence of butyrate, as compared with results in its absence (data not shown), this is presumably due to Ca^{2+} fluxes arising from aggregation and secretion, as originally shown by Massini and Luscher.[30]

There appear to be two major differences between the reactions of platelets to butyrate and those reported with other cells that may shed some further light on the mechanism of its action. First, with platelets, the maximal response to butyrate was seen after 30 to 60 minutes of incubation, whereas with other cells, ~36 hours must elapse before the effects of butyrate are observed: this may reflect the role of platelets as excitable cells. Second, no ultrastructural changes in platelets were seen during the 60-minute incubation and, even in experiments in which platelets were incubated with butyrate for 36 hours, no ultrastructural changes were observed in thin sections beyond the storage-induced changes in controls (data not shown).

Various hypotheses have been proposed to explain the morphological changes resulting from the interaction of butyrate with eukaryotic cells, but these hypotheses have generally implied a direct effect of butyrate on cell structures rather than facilitation of the uptake of cellular effectors. The fact that KB cells do not show morphological changes with butyrate in the presence of actinomycin D, cycloheximide and colchicine[2] was explained as indicating either: (a) a need for RNA synthesis, protein synthesis, and microtubule assembly; or (b) that the cells were not receptive to butyrate when blocked in G1 and G2 phases of the cell cycle by actinomycin D and cycloheximide or in M phase by colchicine. The latter explanation appears to be negated since the cell cycle is not a variable with platelets. Furthermore, platelets are sensitive to changes in microtubule organization,[31] but the absence of morphological change in the presence of butyrate alone suggests that the anion does not have a direct effect on microtubule function. Thus, these platelet data support the necessity for RNA or protein synthesis to induce morphological changes in eukaryotic cells in the presence of butyrate, but further support the suggestion that the primary effects of butyrate do not occur at the nuclear level.

The present results suggest that butyrate reversibly modifies the interaction of certain agonists with platelets in such a way as to increase their uptake so as to develop higher intraplatelet concentrations. By analogy with these observations, the primary mechanism of butyrate in mimicking differentiation of eukaryotic cells may be to facilitate the uptake of endogenous ionophores or other effectors that can cause changes in intracellular Ca^{2+} levels or other aspects of cellular metabolism. In this connection, it may be noted that ionophore A23187 has been reported to prevent butyrate-induced morphological changes in Chinese hamster ovary cells.[32] The enhancing effect of butyrate on the expression of recombinant plasmids in mammalian cells[33] has been suggested as also reflecting Ca^{2+}-mediated events.

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Incorporation of Platelet and Leukocyte Lipoxygenase Metabolites by Cultured Vascular Cells

By Andrew I. Schafer, Hiroshi Takayama, Sheila Farrell, and Michael A. Gimbrone, Jr.

When arachidonic acid metabolism is studied during platelet–endothelial interactions in vitro, the predominant cyclooxygenase end products of each cell type (thromboxane B_2 and 6-keto-prostaglandin-$F_{1\alpha}$, respectively) are essentially completely recovered in the cell-free supernatants of these reactions. In contrast, 50% of 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE), the major lipoxygenase metabolite from platelets, is released into the cell-free supernatant. In investigating the basis of this observation, we have found that platelet lipoxygenase metabolites were generated to the same extent during these coincubations but became rapidly incorporated into the endothelial cells. The endothelial cell-associated 12-HETE was present not only as free fatty acid, but was also incorporated into cellular phospholipids and triglycerides. When purified ^3H-12-HETE, ^3H-5-HETE (the major hydroxy acid lipoxygenase product of leukocytes), and ^3H-arachidonic acid (the common precursor of these metabolites) were individually incubated with suspensions of cultured bovine aortic endothelial cells or smooth muscle cells, different patterns of intracellular lipid distribution were found. In endothelial cells, 12-HETE was incorporated equally into phospholipids and triglycerides, whereas 5-HETE was incorporated preferentially into triglycerides, and arachidonic acid was incorporated into phospholipids. In smooth muscle cells, both 12-HETE and 5-HETE showed more extensive incorporation into triglycerides. The rapid and characteristic incorporation and esterification of platelet and leukocyte monohydroxy fatty acid lipoxygenase products by endothelial and smooth muscle cells suggests a possible physiologic role for these processes in regulating vascular function.

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PLATELETS and vascular endothelial cells respond to various stimuli by liberating free arachidonic acid from membrane phospholipid pools. The free fatty acid is then rapidly converted by the cyclooxygenase pathway to predominantly thromboxane A_2 (TXA_2) in platelets and prostacyclin (PGI_2) in endothelial cells. The common substrates for these eicosanoids of opposing physiologic function are the labile prostaglandin endoperoxides PGG_2 and PGH_2. We have recently reported that stimulated platelets can efficiently transfer endoperoxides to endothelial cells to serve as substrates for PGI_2 production, although platelets are unable to use endothelial endoperoxides transferred in the reverse direction for TXA_2 production.[1] In these experiments, the cyclooxygenase metabolites of platelets and endothelial cells were essentially completely recovered in the cell-free supernatants of the reactions, in agreement with the previous observations of others.[2] Platelet arachidonic acid is metabolized not only by a cyclooxygenase mechanism, but also through a lipoxygenase system which leads to the production of a labile hydroperoxy fatty-acid (12-hydroperoxy-5,8,10,14-eicosatetraenoic acid [HPETE]) and its more stable reduction product, the hydroxy fatty acid 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE). In our platelet–endothelial interaction studies, we observed a marked reduction in the recovery of platelet 12-HETE in the cell-free supernatants of reactions involving both platelets and endothelial cells as compared with reactions with the same number of platelets alone.

The present study was designed to investigate the mechanism whereby the recovery of 12-HETE in the cell-free medium of activated platelets is markedly decreased in the presence of endothelial cells. We present evidence that cultured endothelial cells do not inhibit the platelet lipoxygenase system, but rather rapidly take up the 12-HETE formed by the platelets. Furthermore, 12-HETE becomes incorporated into the phospholipids and triglycerides of endothelial cells. The major leukocyte lipoxygenase hydroxy fatty acid metabolite, 5-HETE, is similarly incorporated into endothelial lipids. We have found that these reactions of platelet 12-HETE and leukocyte 5-HETE are also seen when smooth muscle cells cultured from the same vascular source are substituted for endothelial cells; however, 12-HETE and 5-HETE, as well as arachidonic acid, display different patterns of incorporation into endothelial and smooth muscle cell lipids.

MATERIALS AND METHODS

Materials. ^3H-arachidonic acid (87.4 Ci/mmol), ^3H-12-HETE (40.0 Ci/mmol), ^3H-5-HETE (150 Ci/mmol), and ^3H-leukotriene B_4 (LTB_4) (52.0 Ci/mmol) were obtained from New England Nuclear (Boston). Dulbecco's Modified Eagle's Medium and Dulbecco's phosphate-buffered saline (PBS) were obtained from M.A. Bioproducts (Walkersville, Md); 5,8,11,14-eicosatetraenoic acid (ETYA) was kindly provided by Dr W. E. Scott, Hoffman-LaRoche, Nutley, NJ; sodium arachidonate was obtained from Nu-Chek-Prep, Inc (Elysian, Minn); and 6-keto-$PGF_{1\alpha}$ and TXB_2 were obtained from The Upjohn Co (Kalamazoo, Mich). Bovine albumin, essentially fatty acid-free, was obtained from Sigma Chemical Co, St Louis.

Endothelial and smooth muscle cell cultures. Bovine aortic endothelial cells (BAEC) were isolated from the intimal lining of the thoracic aorta of yearling calves by a modification of published

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techniques[3,4] and characterized by morphologic, immunologic, and biochemical criteria, as previously described.[3,4] A strain of these cells (11-BAEC) was grown to confluency in Dulbecco's Modified Eagle's Medium (DME) containing 10% calf serum and was used in subculture passages 2 through 25. Bovine aortic smooth muscle cells (BASMC) were isolated from explant outgrowths of dissected fragments of calf thoracic aortas using standard techniques. BASMC cultures were grown to confluence in the same culture medium as BAEC cultures and used in subculture passages 2 through 5.

Platelet and platelet lipoxygenase preparations. Venous blood anticoagulated with 13.5% acid citrate dextrose (National Institutes of Health Formula A) was centrifuged at 160 g for ten minutes to obtain platelet-rich plasma (PRP). For preparation of washed platelet suspensions, PRP was adjusted to pH 6.5 with additional acid citrate dextrose and centrifuged at 1,500 g for ten minutes and the platelet pellet was washed in buffer containing 8 mmol/L of Na_2HPO_4, 2 mmol/L of NaH_2PO_4, 5 mmol/L of KCl, 135 mmol/L of NaCl, and 10 mmol/L of EDTA, pH 7.4, and finally resuspended in a modified Tyrode's buffer containing 130 mmol/L of NaCl, 9 mmol/L of sodium bicarbonate, 6 mmol/L of dextrose, 10 mmol/L of sodium citrate, 10 mmol/L of Trizma base, 0.8 mmol/L of KH_2PO_4, 3 mmol/L of KCl, and 0.9 mmol/L of $MgCl_2$, pH 7.3. Platelet counts were determined in a model F Coulter Counter (Coulter Electronics Inc, Hialeah, Fla).

A soluble, cell-free platelet 12-lipoxygenase generating system (PLO) was prepared by separating the supernatant of freeze-thawed lysates of washed platelets after centrifugation at 100,000 g for 60 minutes.[5,6]

Experimental design. Replicate plated cultures of BAEC or BASMC in 10-cm Petri dishes were washed three times with fresh Dulbecco's PBS, incubated at 4 °C for 15 minutes with 0.02% EDTA, and were then gently scrape-harvested with a rubber policeman and washed with and resuspended in PBS, pH 7.4. Washed suspensions of platelets were prepared as described above. Suspensions of cell types alone or in combination were reacted in a final volume of 1 mL with ^{3}H-arachidonic acid (0.2 μCi, 10 μmol/L) and stirred at 37 °C for five minutes. The reaction mixtures were then immediately microfuged in a Beckman microfuge (Beckman Instruments, Inc, Spinco Div, Palo Alto, Calif) at 8,700 g for two minutes. The supernatants were separated, and the cell pellets were resuspended in the original volume of PBS. Supernatant and pellet resuspensions were acidified to pH 3.0 with 2.7 mol/L of citric acid and extracted twice with ethyl acetate in preparation for HPLC.

In order to study the incorporation of purified arachidonic acid and lipoxygenase metabolites into endothelial and smooth muscle cell membrane lipids, 1.0-mL suspensions of cells were incubated with ^{3}H-arachidonic acid (AA), ^{3}H-12-HETE, ^{3}H-5-HETE, or ^{3}H-LTB_4 and were stirred for 15 minutes at 37 °C. At the end of the incubation period, cell suspensions were microfuged, the supernatants were separated, and, where indicated, the cell pellets were washed once with PBS containing 0.5% bovine serum albumin (BSA) (essentially fatty acid-free) and then with PBS without albumin. The washed cell suspensions were then extracted for TLC, as described below.

Thin-layer chromatography (TLC). Incubations were terminated by the addition of 3.75 vol of chloroform/methanol (1:2, vol/vol), followed by the addition of 1 vol each of chloroform and H_2O. The upper methanol/water phase was washed three times with two vol of chloroform, and the chloroform phases were pooled and concentrated under N_2.[7] The extracted lipids were applied to Linear-K (LK5) 20 × 20 cm, 250-μ Silica Gel plates (Whatman, Clifton, NJ), and the phospholipids and neutral lipids were separated with an ascending solvent containing benzene/diethyl ether/ethanol/acetic acid (100.80:4:0.2, vol/vol). Lipids were visualized with I_2, and plates were scraped for scintillation counting of their radioactivity. For free fatty acids, radioactive peaks cochromatographing with authentic standards of arachidonic acid, 12-HETE, 5-HETE, and LTB_4 were counted. The R_x values of 12-HETE and 5-HETE in relation to arachidonic acid were 0.71 and 0.65, respectively, under these chromatography conditions.

HPLC. Pooled ethyl acetate extracts of terminated reactions were evaporated under N_2, and the samples were injected in 50 μL of methanol into a 4.6 × 250 mm Ultrasphere ODS 5-μm reverse-phase HPLC column (Altex Scientific Inc, Berkeley, Calif). Separation of all arachidonic acid metabolites was performed as previously described,[8] using increasing proportions of acetonitrile (30.5% to 95%) in aqueous phosphoric acid (pH 2.0). The flow rate was 1 mL/min The column was equilibrated at 30.5% acetonitrile for 40 minutes before reuse. A Beckman model 110 A single-pump HPLC with a Perkin-Elmer LC-75 variable-wavelength spectrophotometer detector was used, and arachidonic acid metabolites were measured by absorbance at 192 nm and collection of 1.0-mL fractions for scintillation counting of radioactivity. Nonradioactive 6-keto-$PGF_{1\alpha}$, TXB_2, 12-HETE, and arachidonic acid standards were run simultaneously.

Deacylation of phospholipids and triglycerides. To determine if the radiolabel in phospholipids and triglycerides represented esterified 12-HETE or 5-HETE, the phospholipid and triglyceride bands were scraped from TLC plates, eluted, and subjected to alkaline deacylation[9] in 50% methanol containing 0.5 mol/L of KOH. The liberated fatty acids were then applied to and eluted isocratically by reverse-phase HPLC using the solvent system of methanol/water/acetic acid (73:27:0.1) at a flow rate of 1 mL/min, and compared with known standards of 12-HETE and 5-HETE.

Lowry protein analysis. Cell protein was determined by the method of Lowry et al,[10] using BSA as standard.

RESULTS

Washed suspensions of platelets rapidly convert ^{3}H-AA into ^{3}H-thromboxane B_2 (TXB_2), the major platelet cyclooxygenase metabolite, and ^{3}H-12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE), the major platelet lipoxygenase metabolite. These radiolabeled products, as well as unconverted ^{3}H-AA, are detected by HPLC in the extracted supernatant (Table 1). The major ^{3}H-AA oxygenation metabolite of cultured endothelial cells is ^{3}H-6-keto-$PGF_{1\alpha}$, a stable breakdown product of prostacyclin (PGI_2); endothelial

Table 1. Platelet and Endothelial Cell Metabolites of ^{3}H-AA Recovered in Cell-Free Supernatants

Cells in Reaction Mixture	^{3}H-6-keto-$PGF_{1\alpha}$	^{3}H-TXB_2	^{3}H-12-HETE	^{3}H-AA
Platelets	—	37,180	39,252	46,084
Platelets + EC	7,229	33,663	17,256	20,721

AA, arachidonic acid; TXB_2, thromboxane B_2; HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid

Suspensions of equal concentrations of platelets alone or with aortic endothelial cells were incubated for five minutes at 37 °C with ^{3}H-arachidonic acid (0.2 μCi, 10 μmol/L) and then centrifuged at 8,700 g for two minutes; the supernatants were extracted and analyzed for ^{3}H-arachidonic acid metabolites by high-performance liquid chromatography. Data are expressed as counts per minute of ^{3}H-6-keto-$PGF_{1\alpha}$, ^{3}H-TXB_2, ^{3}H-12-HETE, and ^{3}H-AA. Data are mean values from one of three similar experiments.

cells produce no detectable ^{3}H-TXB$_2$ or ^{3}H-12-HETE under our assay conditions. When endothelial cells are coincubated with platelets in suspension and reacted with ^{3}H-AA, the supernatant of the mixed reaction contains > 90% of the ^{3}H-TXB$_2$ found in the supernatant of the reaction containing the same number of platelets alone. In contrast, the supernatant of the endothelial-platelet coincubation contains only 44% of the ^{3}H-12-HETE found in the supernatant of the reaction containing platelets only (Table 1).

These data suggest that either: (a) the presence of endothelial cells selectively inhibits platelet lipoxygenase metabolism; or (b) platelet lipoxygenase metabolites are formed normally in the presence of endothelial cells but become rapidly endothelial cell-bound or incorporated, thus reducing their recovery in the supernatant of the platelet–endothelial cell mixture. In order to examine the latter possibility, mixed cell suspensions of endothelial cells and platelets were incubated with ^{3}H-AA, the completed reactions were centrifuged, and both the supernatant and the cell pellet were extracted for HPLC analysis. As shown in Table 2, almost all of the ^{3}H-TXB$_2$ produced by platelets in coincubation with endothelial cells is released into the supernatant, but 43% of the ^{3}H-12-HETE produced by platelets under these conditions is found associated with the cell pellet. When platelets are stimulated with ^{3}H-AA in the presence of suspensions of smooth muscle cells (cultured from bovine aorta, the same source as endothelial cells), similar data are obtained (Table 2). Although some of the 12-HETE associated with the mixed cell pellets in this experiment may represent incorporation into platelets, the data in Table 1 indicate that platelet 12-HETE or possibly an intermediate precursor of the platelet lipoxygenase pathway is rapidly taken up by the vascular cells.

A PLO rapidly converts ^{3}H-AA completely into ^{3}H-12-HETE.[6] We have previously shown that this reaction can be blocked in the presence of the competitive AA oxygenase inhibitor, ETYA, under these experimental conditions.[6] Table 3 shows that when PLO is stimulated with ^{3}H-AA in the presence of endothelial cells, the extracted endothelial cell pellet contains slightly >50% free fatty acid, and another 30% of the radiolabel is found incorporated into endothelial phospholipids and triglycerides. When ^{3}H-AA conversion to platelet lipoxygenase products is blocked in this incubation by ETYA, 80% of the radiolabel is found incorporated into membrane lipids, mostly into phospholipids. This represents efficient incorporation of unconverted ^{3}H-AA into endothelial cell phospholipids. A similar pattern of incorporation is noted when ^{3}H-AA is incubated with endothelial cells in the absence of PLO, and this is not altered by ETYA. Finally, as shown in Table 3, purified platelet ^{3}H-12-HETE is incorporated into endothelial cell phospholipids and triglycerides to an extent and in a pattern similar to that noted when a PLO stimulated with ^{3}H-AA is used.

These observations suggest that the uptake and incorporation of platelet lipoxygenase metabolites into endothelial cells primarily involves the end-product 12-HETE itself, rather than a lipoxygenase pathway precursor of 12-HETE. Furthermore, the data indicate that endothelial cell-associated 12-HETE is recovered not only as the free fatty acid but can also become incorporated into cellular phospholipids and triglycerides.

In order to examine directly the incorporation of lipoxygenase metabolites into vascular cell lipids, purified platelet ^{3}H-12-HETE, ^{3}H-5-HETE (the major monohydroxy acid lipoxygenase product of leukocytes), ^{3}H-LTB$_4$, and ^{3}H-AA (the common precursor of these metabolites) were individu-

Table 2. Cellular Uptake of Platelet ^{3}H-AA Metabolites During Platelet-Endothelial and Platelet-Smooth Muscle Cell Coincubations

	^{3}H-TXB$_2$	^{3}H-12-HETE
EC + platelets: Supernatant	43,047	22,068
EC + platelets: Pellet	3,082	16,603
Percentage of ^{3}H-pellet–associated	6.7	42.9
SMC + platelets: Supernatant	27,663	21,444
SMC + platelets: Pellet	1,133	23,987
Percentage of ^{3}H-pellet–associated	3.9	52.8

AA, arachidonic acid; TXB$_2$, thromboxane B$_2$; 12-HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid.

Coincubations of platelets and aortic endothelial cells (EC), or platelets and aortic smooth muscle cells (SMC), each containing an approximate platelet-vascular cell ratio of 5:1, in a reaction volume of 1.5 mL. The mixed cell suspensions were incubated for five minutes at 37 °C with ^{3}H-AA (0.2 μCi, 10 μmol/L) and then were centrifuged at 8,700 g for two minutes; the supernatants were separated ("supernatant"). The cell pellets were resuspended in the original volume of reaction buffer ("pellet"). Supernatant and pellet resuspensions were extracted, and platelet metabolites were analyzed by high-performance liquid chromatography: data are expressed as counts per minute of ^{3}H-TXB$_2$ and ^{3}H-12-HETE, and are representative of three similar experiments.

Table 3. Lipid Distribution of AA and Its Platelet 12-Lipoxygenase Metabolites in Cultured Endothelial Cells

	^{3}H Fatty Acid Incorporation cpm (% of total cpm)		
Reaction/Components	Phospholipid	Free Fatty Acid	Triglyceride
1. ^{3}H-AA + EC + PLO	7,461 (18.5)	22,846 (56.6)	4,631 (11.5)
2 ^{3}H-AA + EC + PLO + ETYA	27,856 (69.1)	6,229 (15.5)	4,372 (10.8)
3. ^{3}H-AA + EC	31,091 (77.0)	3,528 (8.7)	4,369 (10.8)
4. ^{3}H-AA + EC + ETYA	29,094 (72.1)	4,956 (12.3)	4,417 (10.9)
5. ^{3}H-12-HETE + EC	5,337 (13.2)	27,711 (68.6)	4,113 (10.2)

AA, arachidonic acid; 12-HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid, EC, endothelial cells; PLO, platelet lipoxygenase preparation.

Equal aliquots of a suspension of EC were reacted with ^{3}H-AA (arachidonic acid, 0.9 μCi) in the presence (reactions 1 through 2) or absence (reactions 3 through 4) of a semipurified soluble PLO. In reaction 2, 30 μmol/L of ETYA was included to block conversion of ^{3}H-AA to radiolabeled lipoxygenase metabolites by the PLO. Reaction 3 was included to control for the effect of ETYA on utilization of ^{3}H-AA by EC. In reaction 5, EC were reacted with ^{3}H-12-HETE (0.2 μCi). Each incubation was terminated after 30 minutes (37 °C), centrifuged at 8,700 g for two minutes, and the cell pellets were extracted for thin-layer chromatography. Data represent counts per minute of scraped bands cochromatographing with phospholipid, free fatty acid, or triglyceride. Data in parentheses represent percentage of total counts per minute in each fraction. Additional radioactivity on plates (to total of 100 percent) represents unidentified products.

ally incubated with suspensions of cultured endothelial cells or smooth muscle cells. At the end of the incubation period, the cells were centrifuged, the cell pellets were washed with buffer containing 0.5% albumin in order to bind and eliminate extracellular free fatty acid trapped in the pellet, washed again with albumin-free buffer, and then extracted for TLC. In most cases, maximal uptake of ^{3}H-12-HETE, ^{3}H-5-HETE and ^{3}H-AA by the endothelial and smooth muscle cells occurs within 15 minutes (Fig 1). Although > 25% of the added ^{3}H-12-HETE, ^{3}H-5-HETE, and ^{3}H-AA becomes endothelial cell-associated within 15 minutes, <5% of the added ^{3}H-LTB$_4$ is taken up by the cell pellets (Table 4 and Figure 1). As shown in Table 4, > 50% of the cell-associated ^{3}H-AA is incorporated into endothelial phospholipids; it is incorporated to a lesser extent into triglycerides. Incorporation of ^{3}H-12-HETE is approximately equally distributed between endothelial phospholipids and triglycerides; a higher percentage of ^{3}H-12-HETE than ^{3}H-AA remains as the free fatty acid. The leukocyte lipoxygenase monohydroxy acid ^{3}H-5-HETE is preferentially incorporated into endothelial triglycerides (> 50%) and to a lesser extent into phospholipids. In contrast, in smooth muscle cells, there is a preferential shift of incorporation of ^{3}H-12-HETE, ^{3}H-5-HETE, and ^{3}H-AA into triglycerides as opposed to phospholipids, a pattern strikingly different from that observed in endothelial cells. In contrast to AA and its monohydroxy fatty acid lipoxygenase products, most of the small amount of cell-associated LTB$_4$ remains as free fatty acid (84% in endothelial cells and 78% in smooth muscle cells). In general, comparable incorporation data were obtained using monolayers rather than suspensions of endothelial and smooth muscle cells; however, there was less uptake of ^{3}H-AA and ^{3}H-12-HETE, and more uptake of ^{3}H-5-HETE into endothelial and smooth muscle cell monolayers than into cell suspensions (data not shown).

Following incubation of endothelial cells with ^{3}H-12-HETE or ^{3}H-5-HETE, the radioactivity incorporated into the phospholipids and triglycerides was isolated on TLC plates, eluted, and subjected to alkaline deacylation. The liberated fatty acids were then identified by HPLC to be unmodified 12-HETE and 5-HETE, respectively.

DISCUSSION

In the course of experiments to study the regulation of AA metabolism during platelet–endothelial cell interactions, we noted a marked loss of recovery of 12-HETE, the major platelet lipoxygenase metabolite, in the cell-free supernatants of incubations involving both cell types. In contrast, there was almost complete recovery in the cell-free medium of the characteristic cyclooxygenase products of platelets and endothelial cells. These observations suggested that either: (a) endothelial cells exerted a relatively selective inhibitory effect on platelet lipoxygenase metabolism; or (b) platelet lipoxygenase metabolites were produced normally in the presence of endothelial cells, but were relatively selectively bound to or incorporated into the endothelial cells, thus reducing their recovery in the cell-free medium. Analysis of the cell pellets of these reactions confirmed the latter explanation. Furthermore, it was found that cultured smooth muscle cells isolated from the same vascular source similarly avidly took up platelet 12-HETE.

Endothelial and smooth muscle cell uptake of 12-HETE was noted when 12-HETE was generated by platelets activated in the presence of cultured vascular cells and also when purified 12-HETE was incubated with cultured vascular cells in the absence of platelets. A similar rapid cellular uptake was seen with 5-HETE, the major monohydroxy fatty acid lipoxygenase product of leukocytes. However, there was considerably less cellular uptake of the leukocyte-derived dihydroxy fatty acid lipoxygenase metabolite, LTB$_4$. We examined the metabolic fate of 12-HETE and 5-HETE that had become associated with endothelial and smooth muscle cells. Some of the cell-associated free hydroxy fatty acid was apparently reversibly bound and could be washed away using

Table 4. Uptake and Lipid Distribution of 12-HETE, 5-HETE, LTB$_4$, and AA in Cultured Endothelial Cells and Smooth Muscle Cells

	Cell-Associated Eicosanoid (Percentage of Total Added cpm ± SEM in Cell Pellets)	Distribution of Cell-Associated Eicosanoids (Fraction of Cell-Associated cpm ± SEM)		
		Phospholipids	Triglycerides	Free Fatty Acid
Endothelial cells				
12-HETE	35.5 ± 8.4	0.35 ± 0.04	0.34 ± 0.02	0.18 ± 0.04
5-HETE	27.7 ± 3.5	0.33 ± 0.02	0.53 ± 0.03	0.08 ± 0.01
LTB$_4$	4.3 ± 1.2	0.14 ± 0.01	0.02 ± 0.01	0.84 ± 0.01
AA	31.5 ± 2.2	0.61 ± 0.04	0.37 ± 0.04	0.02 ± 0.01
Smooth muscle cells				
12-HETE	13.6 ± 1.6	0.27 ± 0.03	0.59 ± 0.01	0.10 ± 0.02
5-HETE	14.8 ± 1.9	0.17 ± 0.02	0.77 ± 0.02	0.04 ± 0.01
LTB$_4$	3.7 ± 0.7	0.16 ± 0.03	0.06 ± 0.01	0.78 ± 0.02
AA	35.8 ± 6.0	0.52 ± 0.02	0.47 ± 0.02	0.01 ± 0.01

HETE, hydroxy-5,8,10,14-eicosatetraenoic acid; LTB$_4$, leukotriene B$_4$; AA, arachidonic acid.

Suspensions of bovine aortic endothelial cells (3.3 × 10^6 cells per milliliter, 0.54 mg protein per milliliter) or bovine aortic smooth muscle cells (2.1 × 10^6 cells per milliliter, 0.41 mg protein per milliliter) were reacted with ^{3}H-12-HETE, ^{3}H-5-HETE, ^{3}H-LTB$_4$, or ^{3}H-AA for 15 minutes at 37 °C. Terminated reactions were centrifuged at 8,700 g for two minutes and washed in succession with phosphate-buffered saline (PBS) + 0.5% albumin and PBS without albumin. The washed cell pellets were extracted for thin-layer chromatography, as described in the Methods section. Data are expressed as the percentage of total added counts per minute (cpm) remaining associated with the washed cell pellets and the fraction of cell-associated cpm distributed to phospholipids, triglycerides, and free fatty acids, representing means ± SEM of duplicate determinations in two separate experiments.

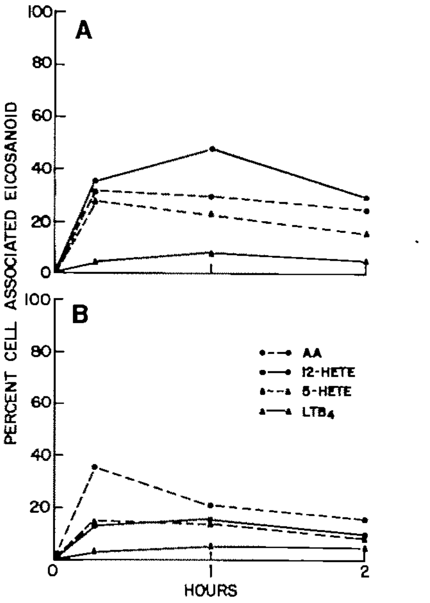

Fig 1. Suspensions of (A) endothelial and (B) smooth muscle cells were reacted with ^{3}H-12-hydroxy-5,8,10,14-eicosatetraenoic acid (HETE), ^{3}H-5-HETE, ^{3}H-leukotriene B_4, or ^{3}H-arachidonic acid at 37 °C, as described in Table 4. At different time periods, aliquots were withdrawn, centrifuged at 8,700 g for two minutes and washed in succession with phosphate-buffered saline (PBS) + 0.5% albumin and PBS without albumin. Data are expressed as percentage of added radioactivity remaining cell associated after washing, and represent the mean of duplicate determinations in two separate experiments.

a buffer containing albumin. More than 80% of the remaining cell-associated 12-HETE or 5-HETE, however, was found to be incorporated into cellular phospholipids and triglycerides in both endothelial and smooth muscle cells. In contrast, ~80% of the small amount of cell-associated LTB_4 remained as free fatty acid.

Furthermore, 12-HETE, 5-HETE, and their common precursor, AA, displayed distinct and characteristic distribution within the lipids of vascular cells. In endothelial cells, 5-HETE was preferentially incorporated into triglycerides, arachidonic acid into phospholipids, and 12-HETE approximately equally into triglycerides and phospholipids. In smooth muscle cells, there was a striking shift of incorporation of all three fatty acids into triglycerides over phospholipids. This was most prominent with 5-HETE, which incorporated 77% into triglycerides and only 17% into phospholipids. In contrast to the monohydroxy fatty acids and arachidonic acid, leukotriene B_4, a dihydroxy fatty acid product of leukocyte lipoxygenase, was not taken up by either endothelial cells or smooth muscle cells. It thus appears that phospholipid and triglyceride esterification is specific for monohydroxy fatty acids in vascular cells, since other oxygenated metabolites of arachidonic acid are not taken up and incorporated. Stenson and Parker[11] initially demonstrated that hydroxylated fatty acids can be incorporated into cellular triglyceride and phospholipids by showing esterification of 12-HETE into human neutrophil membrane lipids. They also showed that 5-HETE, a lipoxygenase product of neutrophils, was likewise esterified into the membrane phospholipids and triglycerides of its cell of origin.[12] Subsequently, the uptake and incorporation of monohydroxy fatty acid lipoxygenase products of platelets and leukocytes into cellular lipids was reported with mouse peritoneal macrophages[13] and a mouse macrophage-like tumor cell line.[14] In the latter study, consistent with our findings using cultured vascular cells, leukotriene B_4 was not esterified into cellular lipids.[14] In a recent abstract, Frawley et al have reported incorporation of 5-HETE into phospholipids and neutral lipids of human umbilical vein endothelial cells.[15] However, in contrast with our observations, the uptake of 5-HETE was relatively slow (during five hours) and other monohydroxy fatty acids, including 12-HETE, were not taken up. The basis for the differences in these findings is unclear at the present time.

Our finding that AA, 12-HETE, and 5-HETE display different patterns of intracellular lipid distribution in cultured bovine aortic endothelial cells and smooth muscle cells suggests that the enzymes involved in fatty acid esterification may have substrate specificity.[14] The striking shift to triglyceride incorporation of all three fatty acids in smooth muscle cells in comparison with endothelial cells may be a function of differences in the major lipid subclass composition of different types of cultured vascular cells.[16] These findings may also indicate a cell-specific role for triglyceride formation and turnover in fatty acid metabolism. Accumulation of fatty acids in triglycerides has been previously shown in guinea pig aortic smooth muscle cells,[17] as well as in cultured bovine aortic endothelial cells,[18] and other types of cultured cells.[19-22] It has been suggested that AA incorporation into triglycerides may serve as a temporary storage site, and AA contained in triglycerides may be mobilized and transferred to phospholipids when the metabolic need arises.[18] The role of hydroxylated fatty acids in membrane phospholipids and triglycerides, however, has not been determined.

Our findings have several implications. First, the potential for avid uptake and incorporation of monohydroxy fatty acids into endothelial and smooth muscle cells is important to consider when investigating their lipoxygenase metabolism, either separately or in combination with other cell types in vitro. Second, uptake of monohydroxy fatty acid lipoxygenase products by vascular cells in vivo may represent a means of terminating the biological activity of these eicosanoids.[15] Finally, the rapid uptake of 12-HETE and 5-HETE and their unique patterns of cellular lipid distribution suggests a possible physiologic role for these reactions in regulating vascular function. The lipid composition of vascular cells can be modified by exposure to different free fatty acids in the surrounding medium,[16,23,24] and these alterations can affect certain metabolic processes, such as prostacyclin synthesis.[25] Our observations raise the possibility that specific lipoxygenase products, generated by activated platelets and leukocytes, can locally influence vessel wall function through their incorporation into endothelial and smooth muscle cell lipids.

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The Pattern of HLA-DR and HLA-DQ Antigen Expression on Clonable Subpopulations of Human Myeloid Progenitor Cells

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Three subpopulations of human myeloid progenitor cells (CFU–GM) can be distinguished by differences in their kinetics of development; the liquid phase pre-CFU–GM, the day 14 CFU–GM, and the day 7 CFU–GM. The relative cell membrane densities of the HLA-DR and HLA-DQ antigens expressed by the three subpopulations was investigated by comparing the amount of antibody required to deplete bone marrow cell preparations of each cell type. Three separate approaches were used—complement (C′) cytotoxicity, antiglobulin/C′-cytotoxicity and immune rosette depletion. Similar results were obtained for all three procedures, although the latter two gave a tenfold greater sensitivity over the standard C′-cytotoxicity method. At saturating anti-HLA-DR antibody concentrations, 85% to 95% of cells within the three myeloid subpopulations were found to express HLA-DR antigens. However, the relative amount of HLA-DR expressed by these subpopulations increased from the pre-CFU–GM to the day 7 CFU-GM. The expression of HLA-DQ antigens was considerably lower and could only be detected by using the more sensitive procedures. Only 50% of day 7 and 14 CFU-GM progenitor cells expressed detectable HLA-DQ antigens, whereas a greater proportion (80%) of the pre-CFU-GM were HLA-DQ positive. The pattern of HLA-DQ expression on these clonable precursors was quite distinct and opposite to the cell membrane density of the HLA-DR antigens. Because these three progenitor cell populations are thought to be linked in differentiation sequence, these results provide indirect support for the hypothesis that HLA class II antigens are implicated in regulatory mechanisms during normal myeloid cell differentiation.

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THE HUMAN MAJOR histocompatibility complex (MHC) codes for three distinct class II antigens, the HLA-DR, DQ (DC), and DP (SB) molecules,[1] which are known to function as regulators in the immune response. These membrane antigens have a restricted cell distribution, being expressed predominantly by B lymphocytes, monocytes, and activated T lymphocytes.[2] It is therefore of interest that hemopoietic progenitor cells of the myeloid and erythroid lineages also express at least one type of class II antigen at their cell membranes.[3–5] These antigens are not found on the mature myeloid and erythroid cells, thus raising the intriguing possibility that class II antigens may also function as regulators of early events during hemopoiesis. Indeed, it has been shown that activated T lymphocyte-mediated inhibition of the more immature, clonable erythroid progenitors (BFU-E) is HLA-DR restricted.[6] Furthermore, the expression of class II antigens on myeloid colony forming cells (CFU-GM) coincides with the ability of prostaglandin E and acidic isoferritins to inhibit in vitro colony formation.[7,8]

Human myeloid progenitor cells which form colonies of mature progeny in cell culture can be divided into at least three subpopulations based on differences in physical properties, response to stimuli, and kinetics of development.[9–11] This last characteristic is the one most commonly used to distinguish between these immature cell types, which are thought to be linked in a differentiation sequence. Thus, the pre-CFU-GM are the more immature progenitors which give rise to the day 14 CFU-GM, which in turn generate the more mature day 7 CFU-GM.[9] The expression of HLA-DR antigens on these subpopulations of CFU-GM progenitor cells has been confirmed by several investigators, although some discrepancies exist. A number of investigators have demonstrated that 90% or more of the CFU-GM progenitor cell population express HLA-DR antigens,[12–14] whereas others have reported that only 50% are HLA-DR positive.[7,8] Similar inconsistencies have occurred for the more immature progenitor cells, the pre-CFU-GM, and the mixed CFU-GEMM colonies.[8,15–18]

More recently, the pattern of HLA-DQ and DP antigen expression on hemopoietic progenitor cells has been studied. Compared with HLA-DR expression, HLA-DP molecules are only detected on a proportion of progenitor cells,[19] whereas HLA-DQ molecules are expressed at very low levels, if at all.[19–21]

In studying the patterns of class II antigen expression on hemopoietic progenitor cells, most reports have used antibodies at saturating levels. However, by comparing the amount of antibody required to deplete the different CFU-GM progenitor cell populations, information concerning the relative membrane densities of the antigen can be gained. This in turn may provide insight to the sequence of events in myeloid cell differentiation. Using this approach with a rabbit polyclonal anti-class II antigen serum, Moore et al[15] found that the pre-CFU-GM progenitor cell population expressed the greatest abundance of class II antigen, followed by the day 14 CFU-GM, whereas the day 7 CFU-GM expressed the least. In contrast, Broxmeyer,[8] using a monoclonal antibody (mcAb) to class II antigens, showed the reverse pattern—the pre-CFU-GM progenitor cell population appeared not to express class II antigens, whereas the day 7 CFU-GM expressed the most.

In this present study, we have established the titration curves of several anti-class II antigen mcAbs against the day 7 and 14 CFU-GM and pre-CFU-GM progenitor cells. Thus, using two anti-HLA-DR mcAbs, the pattern of expression reported by Broxmeyer[8] was confirmed—the only difference being that a higher proportion (85% to 95%) of

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progenitor cells from the three myeloid subpopulations expressed HLA-DR. By using sensitive procedures, HLA-DQ antigens were detected on a proportion of all three subpopulations. However, the cell membrane density of the HLA-DQ antigens was found to decline with cell maturation, which is exactly the reverse of the pattern of HLA-DR expression.

MATERIALS AND METHODS

Antisera. The B5.1 and F5C9 mcAbs have been described elsewhere.[22] The B5.1 mcAb is of the IgG2b subclass, and binds C′; it recognizes a monomorphic epitope expressed on DR molecules (personal communication, M. Steel, Co-ordinator, First International Workshop on Monoclonal Antibodies to Human MHC Class II Antigens, Edinburgh, Aug 28–Sept 2, 1983). The F5C9 mcAb is of the IgG2a subclass, binds C′ and recognizes an epitope expressed on DQ molecules (M. Steel, personal communication). High titred F5C9 and B5.1 antisera obtained from hybridoma-bearing mice were used in this present study. Another monomorphic anti-HLA class II mcAb NEI-011 (also called 7.2[23]) was purchased from New England Nuclear, Boston. It is of the IgG2b subclass, binds C′, and is supplied as a protein A-affinity purified solution at 1 mg protein per milliliter (>95% homogeneity). McAbs used as controls were the monomorphic anti-HLA class I antibody, 246 B8[24] which is of the IgG2a subclass and binds C′; the 19A mcAb (a gift of Dr H Zola, Flinders Medical Centre, Bedford Park, South Australia) which recognizes a human common leukocyte determinant and is non-C′ fixing, and an anti-human T200 glycoprotein mcAb, F2.5.[25]

The sheep anti-mouse immunoglobulin serum (SAMG) was a generous gift of Professor I F. C. McKenzie, Department of Pathology, University of Melbourne. The immunoglobulin fraction of the antiserum was isolated by ammonium sulfate precipitation using standard procedures.

Bone Marrow Cells. Sternal bone marrow specimens were obtained, with informed consent, from patients undergoing thoracic/cardiac surgery. Low-density marrow leukocytes were isolated on a Ficoll-Paque gradient (Pharmacia, Uppsala, Sweden), washed and resuspended in Dulbecco's Modified Eagles Medium (DME; Flow Laboratories, McLean, Va) containing 5% heat-inactivated bovine serum.

Antibody-specific Cell Depletion Procedures

All procedures used 17 × 100 mm plastic tubes (Falcon, Oxnard, Calif).

C′-dependent cytotoxicity. Bone marrow leukocytes (1×10^6) in 0.4 mL were mixed with an equal volume of antibody or normal mouse serum (NMS) at appropriate dilutions and were incubated for 30 minutes at room temperature (RT). The cells were washed and resuspended in 0.8 mL of a 1.2 dilution of rabbit C′ (preselected for low nonspecific toxicity; Commonwealth Serum Laboratories, Melbourne, Australia) or heat-inactivated serum (for controls), and incubated a further 60 minutes at 37 °C. Controls included cells incubated with: no antibody (NMS) ± C′, anti-HLA class I mcAb 246 B8 ± C′ (positive C′ control), and test antibody without C′.

Anti-globulin/C′-cytotoxicity. Bone marrow cells were treated as above except that, following the first antibody incubation, 1 mg SAMG (optimal concentration) was added. The cells were incubated for ten minutes at RT and washed and resuspended in rabbit C′ as described. The non-C′ binding 19A mcAb was included as a control to assess the effectiveness of second antibody binding.

Immune rosette depletion. Bone marrow cells (1×10^6) were incubated with antibody as above, washed three times, and resuspended in 0.5 mL of DME/5% serum. Controls included cells incubated with NMS or 246 B8 mcAb. Rosette formation was performed as described elsewhere.[26] Briefly, SAMG was coupled to sheep erythrocytes by chromic chloride, and 0.1 mL of a 2% erythrocyte suspension was added to the bone marrow cells. After a gentle centrifugation at 75 g for three minutes, the cell mixture was incubated for 30 minutes at RT to allow rosette formation. The cell pellet was then carefully resuspended in its supernatant and 0.4 mL of Ficoll-Paque was underlaid. Following centrifugation at 200 g for five minutes, the nonrosetted (interface) cells were collected.

For all procedures, the cells were given a final wash and divided for subsequent CFU-GM assays.

Myeloid Progenitor Cell Colony Assays

A 50% nonspecific loss of cells during the antibody-cell depletion procedures was assumed. Half the cells ($\sim 2.5 \times 10^5$ total for control tubes) from each tube were assayed for pre-CFU-GM, while the remaining half were equally divided into six cultures ($\sim 4 \times 10^4$ cells/culture) for day 7 and 14 CFU-GM.

Day 7 and 14 CFU-GM. For soft agar culture, DME containing the following supplements was used as the plating medium: 2mmol/L of glutamine, 1 mmol/L of sodium pyruvate, 0.8% 50 × minimum essential amino acid mixture, 0.6% 100 × nonessential amino acid mixture, 16 μg/mL L-asparagine, 8 μg/mL L-serine, 0.6% vitamin mixture, 100 IU/mL penicillin, 100 μg/mL streptomycin, 0.12% sodium bicarbonate (all from Flow), 10^{-5} mol/L of 2-mercaptoethanol, 15% fetal calf serum (FCS, Commonwealth Serum Laboratories) and 0.3% bactoagar (Difco, Detroit). The cells were diluted in 6.5 mL of plating medium, containing an optimal concentration of colony-stimulating activity (CSA), and then equally divided into six 35-mm plastic culture dishes (Kayline, Adelaide, South Australia). The cultures were incubated at 37 °C in a humidified 10% CO_2 atmosphere; three plates were scored for colonies (> 50 cells) after seven days, and the remaining three were scored after 14 days of incubation.

Pre-CFU-GM. Pre-CFU-GM progenitor cells can be defined by their ability to give rise to CFU-GM following incubation in liquid suspension culture.[27] In 17 × 100 mm Falcon 2057 tubes, cells ($\sim 2.5 \times 10^5$) were suspended in 1 mL of DME containing 15% FCS, 10^{-5} mol/L of 2-mercaptoethanol and 10% CSA. After five days of suspension culture (maximal CFU-GM expansion was found to occur after four to five days of liquid culture and then to decline), 0.3 mL of cells was taken and plated in triplicate CFU-GM soft-agar cultures containing CSA, as described above. The plates were incubated for a further seven days, and CFU-GM colonies were scored. Under these conditions, expansion of the CFU-GM numbers ranged from 160% to 224% (mean 182%) as compared with day 7 CFU-GM numbers obtained without prior liquid culture

Sources of CSA. Conditioned medium (CM) from the human fibroblast cell line 5637[28,29] was routinely used as a source of CSA. For the preparation of 5637-CM, cells were grown in DME/5% bovine serum containing Cytodex-3 beads (Pharmacia, Uppsala, Sweden). After five days of culture, the CM was pooled, concentrated fivefold, and dialyzed against 5 vol of deionized-distilled water using an Amicon TCF Thin Channel System with a YM10 filter (Amicon, Lexington, Mass). Following sterilization through a 0.2-μm filter, the 5637-CM was stored at −20 °C where it retained biological activity for at least six months. The optimum concentration of various batches of 5637-CM was found to be 5% to 10% for cultures containing 5×10^4 cells per milliliter (day 7 CFU-GM numbers, 52 ± 15, mean + SEM, n = 40). For some experiments, as indicated, CM from the human macrophage cell line GCT[30] (GIBCO, Grand Island, NY) was used at a 10% final concentration

RESULTS

Pattern of expression of the HLA-DR antigens. Figure 1A shows the results of a representative experiment (one of four separate experiments) in which the anti-HLA-DR mcAb B5.1 was titrated for inhibition of CFU-GM colony formation in the C′-dependent cytotoxicity assay. At high concentrations of mcAb (1:100 dilution), colony formation for the three CFU-GM progenitor cell compartments (day 7 and 14 CFU-GM and pre-CFU-GM) was inhibited by 85% to 95%, thus confirming the results of others.[12–14] However, at lower concentrations of mcAb, the sensitivities of the CFU-GM compartments diverged, with the day 7 CFU-GM progenitor cells being the most sensitive to anti-HLA-DR/C′ treatment, followed by the day 14 CFU-GM, whereas the pre-CFU-GM progenitor cells were the least sensitive. This was evidenced by the 50% titration points which show, in Fig 1A for example, that the day 7 CFU-GM titrated at a B5.1-antibody dilution of 2×10^{-4}, the day 14 CFU-GM at 10^{-4}, whereas the pre-CFU-GM had the lowest titer at 0.3×10^{-4}. Thus, a tenfold increase (range 5 to 15) in the expression of HLA-DR antigens was demonstrated for the day 7 CFU-GM as compared with the pre-CFU-GM. This pattern of HLA-DR expression is similar to that described by Broxmeyer,[8] the only difference being in the proportion of progenitor cells expressing detectable HLA-DR antigens at saturating antibody levels.

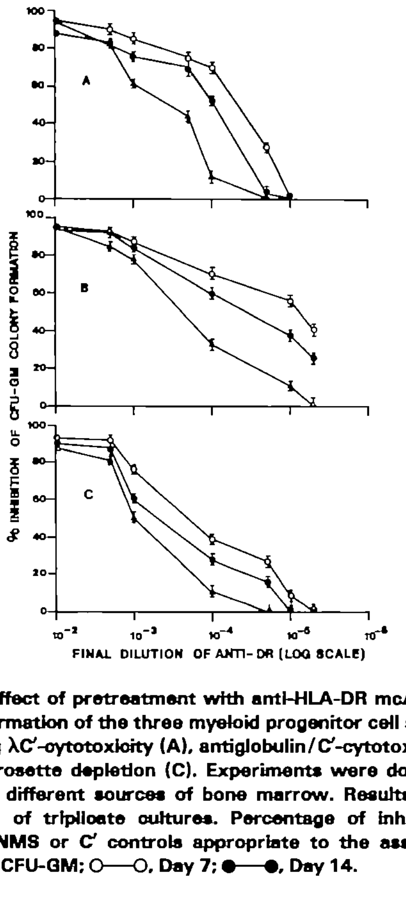

Fig 1. Effect of pretreatment with anti-HLA-DR mcAb (B5.1) on colony formation of the three myeloid progenitor cell subpopulations using C′-cytotoxicity (A), antiglobulin/C′-cytotoxicity (B), or immune rosette depletion (C). Experiments were done separately using different sources of bone marrow. Results are the mean ± SE of triplicate cultures. Percentage of inhibition is relative to NMS or C′ controls appropriate to the assay used. △—△, PRE CFU-GM; ○—○, Day 7; ●—●, Day 14.

To ensure that this pattern of HLA-DR antigen expression was not attributed to the C′ binding characteristics of the C′-cytotoxicity method used, the titration curves were established using two different procedures—anti-globulin/C′-cytotoxicity and immune rosette depletion. The representative experiments shown in Figs 1B and C indicate that the same pattern of HLA-DR antigen expression was obtained regardless of the procedure used. Identical results were found using a second anti-class II antigen mcAb, NEI-011 (data not shown).

To compare the sensitivities of the three procedures, B5.1 titration curves for the day 7 CFU-GM progenitors were set up using the same source of bone marrow. The results showed that both the antiglobulin/C′cytotoxicity and immune rosette depletion procedures have a tenfold greater sensitivity than the standard C′-cytotoxicity method (Table 1). This is in agreement with previous findings.[26,31]

Effect of a different source of CSA. Subclasses of human CSAs have been described which appear to influence different biological properties of CFU-GM.[9,10] For these present experiments, 5637-CM was routinely used as a source of CSA. Morphological analysis of the colony types supported by 5637-CM has not indicated any marked difference compared to other CSAs (Sparrow and Williams, unpublished observations). However, since previous reports investigating the expression of class II antigens on CFU-GM progenitor cells have not used 5637-CM, the titration curves were repeated using GCT-CM as an alternative source of CSA. The same pattern of HLA-DR expression, using the NEI-011 mcAb, was obtained for both GCT-CM and 5637-CM (Fig 2), indicating that the results are not attributed to a unique activity in 5637-CM.

Pattern of expression of the HLA-DQ antigens. Experiments similar to those described above were set up using the anti-HLA-DQ mcAb, F5C9.[22] This antibody has previously been found to have strong activity against normal peripheral blood B lymphocytes in the C′-dependent cytotoxicity. Initial experiments using this same procedure indicated very little (0% to 10%) myeloid colony inhibitory activity, even at high concentrations (1:100 dilution) of the F5C9 mcAb. These results are in agreement with those of others, suggesting that the HLA-DQ antigens are weakly expressed by the CFU-GM progenitors.[19–21]

However, a different set of results was obtained when the more sensitive methods, the antiglobulin/C′-cytotoxicity or

Table 1. Comparison of Sensitivities of Three Assay Procedures Used to Determine Expression of HLA-DR Antigens on Day 7 CFU-GM Progenitor Cells

B5 1 mcAb Dilution	Inhibition (%) of CFU-GM after Treatment by* C′-cytotoxicity	Antiglobulin/ C′-cytotoxicity	Rosette Depletion
1:10^2	95 ± 1	97 ± 2	94 ± 1
1:10^3	83 ± 2	95 ± 2	96 ± 2
1:10^4	63 ± 6	73 ± 3	85 ± 3
1:10^5	24 ± 5	59 ± 2	61 ± 3
1:10^6	8 ± 4	28 ± 4	33 ± 4

*Results are the mean ± SE of triplicate cultures.

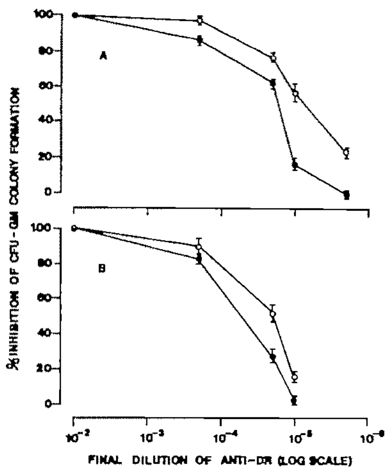

Fig 2. Comparison of colony-stimulating activities from GCT-CM (A) and 5637-CM (B) on bone marrow cells pretreated with anti-HLA-DR mcAb (NEI-011) and C′. Experiments were done separately using different sources of bone marrow. Results are the mean ± SE of triplicate cultures. Percentage of inhibition is relative to C′ controls. O——O, Day 7; ●——●, Day 14.

immune rosette depletion, were used. At high concentrations of F5C9 mcAb, ~50% (range 42% to 72%) of day 7 and 14 CFU-GM were found to express HLA-DQ antigens, whereas an even greater proportion (75% to 85%) of the pre-CFU-GM were HLA-DQ antigen positive (Fig 3). Information from the antibody titration curves suggests that the level of HLA-DQ antigen expression decreases with increasing maturation such that the pre-CFU-GM progenitor cells express the greatest level, followed by the day 14 CFU-GM, whereas the day 7 CFU-GM progenitors express the least. Comparison of the 40% titration points, for example, indicates a 50-fold (range 25 to 200) decrease in HLA-DQ antigen expression by the day 7 CFU-GM relative to the pre-CFU-GM progenitors. This pattern of expression is the reverse of that found for the HLA-DR antigens.

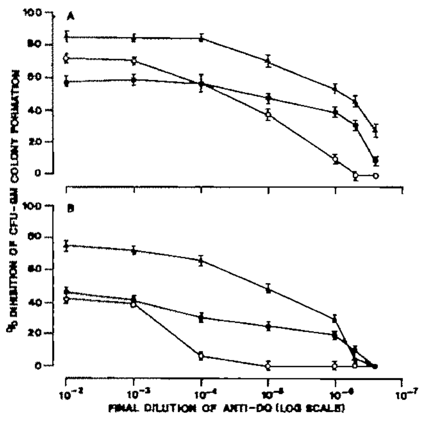

Fig 3. Effect of pretreatment with anti-HLA-DQ mcAb (F5C9) on colony formation of the myeloid progenitor cell subpopulations using antiglobulin/C′-cytotoxicity (A) or immune rosette depletion (B). Different sources of bone marrow were used for each experiment. Results are the mean ± SE of triplicate cultures. Percentage of inhibition is relative to the appropriate NMS or C′ controls. Δ——Δ, PRE CFU-GM; O——O, Day 7; ●——●, Day 14.

Patterns of expression of non-HLA class II antigens. To show that technical manipulations were not accountable for the patterns of HLA-DR and DQ antigen expression presented here, titration curves were established using two mcAbs which recognize different and unrelated cell membrane antigens. The 246 B8 mcAb recognizes a monomorphic epitope on HLA class I antigens, whereas the F2.5 mcAb recognizes the human homologue of the murine T200 glycoprotein (or leukocyte-common antigen). For both antibodies, no significant difference was observed between the titration curves for day 7 and day 14 CFU-GM progenitor cells (Fig 4). Thus, unlike the HLA-DR and DQ molecules, the expression of the HLA class I and T200 antigens is static during this period of myeloid cell differentiation.

DISCUSSION

In this study, the cell membrane density of the HLA-DR antigens was shown to increase with myeloid progenitor cell maturation. An average tenfold increase in the expression of HLA-DR antigens by day 7 CFU-GM progenitor cells as compared with the pre-CFU-GM was demonstrated. These results confirm those of Broxmeyer,[8] although they vary with respect to the observed maximum levels of HLA-DR antigen expression. Broxmeyer found, even at high anti-HLA-DR mcAb concentrations, that only 50% of the day 7 and 14 CFU-GM progenitors expressed the antigen, whereas the pre-CFU-GM appeared not to express HLA-DR at all.[8] Our results show that 85% to 95% of cells from the three myeloid progenitor cell compartments studied express HLA-DR antigens. Presumably the variance in results between those reported here and those reported by Broxmeyer can be attributed to the different sources of complement used, an effect recently illustrated by Falkenburg et al,[32] who showed

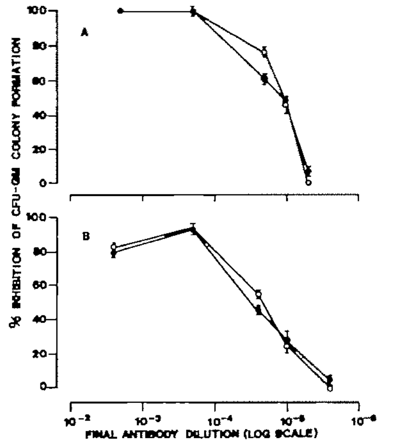

Fig 4. Comparison of inhibition of day 7 and 14 CFU-GM colony formation following pretreatment with: (A) anti-HLA class I mcAb, 246 B8 and C′-cytotoxicity; or (B) anti-T200 glycoprotein mcAb, F2.5 and antiglobulin/C′-cytotoxicity. Results are the mean ± SE of triplicate cultures. Percentage of inhibition is relative to the appropriate C′ controls. O——O, Day 7; ●——●, Day 14.

that hemopoietic progenitor cells differ in their sensitivities to complement. However, the use of complement-dependent procedures in this report does not seem to have biased the results in any way, because similar results were obtained using a noncomplement procedure, immune rosette depletion.

The demonstrable increase in the level of HLA-DR antigen expression during CFU-GM progenitor cell maturation supports the hypothesis that these antigens have a biological function in hemopoiesis. The exact nature of such a role remains unknown. An association between HLA-DR expression and modulation of myeloid differentiation by prostaglandin E and acidic isoferritins has been suggested.[8,17] However, in contrast to our data and those of other investigators,[12-14,19,21] these authors found that only 50% of the CFU-GM progenitor cells expressed HLA-DR; thus, the correlations observed must remain uncertain.

It could be argued that the observed increase in HLA-DR expression may not be unique but could be a common occurrence for cell membrane proteins expressed by the CFU-GM progenitor cells. Evidence against this, however, is that the expression of the separately regulated HLA class I and T200 glycoproteins remains constant during the three stages of myeloid cell differentiation studied. Furthermore, the expression of the HLA-DQ antigens was found to decline with cell maturation.

Of particular interest in this study was the demonstrated pattern of HLA-DQ antigen expression by myeloid progenitor cells. At saturating antibody levels, ~50% of the day 7 and 14 CFU-GM progenitor cells expressed detectable HLA-DQ antigens, whereas an even greater proportion (80%) of pre-CFU-GM progenitors were HLA-DQ positive. Comparison of the titration curves for the three cell populations indicated that the pre-CFU-GM expressed 50 times more HLA-DQ (range 25 to 200) than did the day 7 CFU-GM. This pattern of expression is the reverse to that found for the HLA-DR antigens (see Fig 5 for a diagrammatic representation).

These results could only be obtained when very sensitive procedures were used. The anti-HLA-DQ mcAb F5C9 used in this study is strongly active in the C′-cytotoxicity assay when B lymphocytes are used as target cells.[33] The fact that very little F5C9-binding could be detected on myeloid progenitor cells using the C′-cytotoxicity method suggests that the cell membrane density of the HLA-DQ antigens is comparatively low or that the antibody affinity is lower for myeloid HLA-DQ antigens than for B lymphocyte antigens. This may explain the results of other investigators who were unable to demonstrate HLA-DQ antigen expression by CFU-GM.[19-21] Falkenburg et al[21] used a conventional anti-DQw1 alloantisera with the C′-cytotoxicity procedure. FACS has also been used.[19,20] However, this procedure is limited by the fact that the bone marrow myeloid cells display high nonspecific staining. To overcome this problem, the threshold for positive sorting was raised, reducing the overall sensitivity of the procedure.[20] Linch et al[19] used immune rosette depletion, but their definition of a day 7 CFU-GM colony varied considerably from ours, and thus the results are not directly comparable.

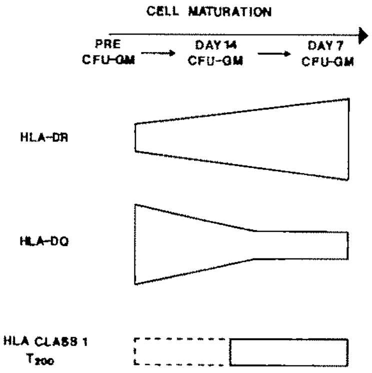

Fig 5. Diagrammatic representation of the changes in cell membrane densities of the HLA-DR and DQ antigens expressed during myeloid cell maturation from the pre-CFU-GM to the more mature day 7 CFU-GM. The expression of other cell surface antigens, such as the HLA class I and T200 glycoproteins remains constant (broken lines represent extrapolated results).

We are currently pursuing the investigation of HLA-DQ antigens on hemopoietic cells. It is possible that the apparent decrease in HLA-DQ antigen expression may result from increased glycosylation of the molecule such that the F5C9 mcAb can no longer recognize its reactive determinant. No data is available to suggest that this is the case. The biologic function of the HLA-DQ antigens on myeloid progenitor cells is unknown. However, in the immune system, HLA-DQ antigens predominantly modulate functions involving the production of suppressor and/or cytotoxic T lymphocytes, whereas the HLA-DR antigens are directly implicated in helper T lymphocyte function.[34] It is therefore tempting to speculate that if similar roles are played by these antigens in hemopoiesis, the decrease in HLA-DQ and corresponding increase in HLA-DR expression seen during myeloid progenitor cell maturation reflect changes in regulatory mechanisms.

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Fibrinogen Binding to Human Blood Platelets: Effect of γ Chain Carboxyterminal Structure and Length

By Ellinor I. B. Peerschke, Charles W. Francis, and Victor J. Marder

Recent evidence suggests that fibrinogen binding to platelets is mediated by the 12 carboxyterminal amino acid residues of the γ chain. Because human plasma fibrinogen γ chains differ in mol wt and carboxyterminal amino acid sequence, we examined the effect of such γ chain heterogeneity on platelet–fibrinogen interactions, using two fibrinogens of distinct composition, separated by ion exchange chromatography. One fibrinogen possessed only γ chains of mol wt 50,000 ($F^{\gamma 50}$), the predominant γ chain species found in plasma. The other fibrinogen possessed equal amounts of γ chains with mol wt 50,000 and 57,500 ($F^{\gamma 50,57.5}$), with the longer γ chain (γ57.5) possessing an amino acid extension at the carboxyterminal end. The latter fibrinogen was 50% less effective than $F^{\gamma 50}$ in supporting ADP-induced platelet aggregation at concentrations of .01 to 2 mg/mL. Scatchard analysis revealed no difference in the binding affinities of the two fibrinogens to ADP-treated platelets, but the amount of $F^{\gamma 50,57.5}$ that was bound to platelets at saturation was only 50% that of $F^{\gamma 50}$. Fibrinogen receptors that remained unoccupied in the presence of saturating concentrations of $F^{\gamma 50,57.5}$, however, could be occupied by fresh $F^{\gamma 50}$. Excess unlabeled $F^{\gamma 50}$ displaced both radiolabeled fibrinogens from activated platelets, and both fibrinogens bound to the same platelet receptor, as judged by the inhibition of binding to stimulated platelets by a monoclonal antibody directed against the glycoprotein (GP) IIb/IIIa complex. Furthermore, an intact GPIIb/IIIa complex was required for these reactions, since platelets incubated with EDTA at 37 °C at alkaline pH failed to aggregate and bound neither fibrinogen in response to ADP following recalcification. Approximately 50% of each fibrinogen bound irreversibly to platelets after one hour and failed to dissociate in the presence of 10 mmol/L of EDTA or excess unlabeled $F^{\gamma 50}$. The data demonstrate that heterodimeric $F^{\gamma 50,57.5}$ binds less well to platelets and supports platelet aggregation only half as well as homodimeric $F^{\gamma 50}$. These results support prior conclusions that the carboxyterminal portion of the γ chain is important in platelet-fibrinogen interactions, and suggest that the 20 amino acid, hydrophobic γ chain carboxyterminal extension of $F^{\gamma 50,57.5}$ may sterically hinder the interaction of this fibrinogen with platelet receptors.

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HUMAN plasma fibrinogen γ chains vary not only with respect to charge[1-4] and sialic acid content,[5] but also to mol wt.[6-11] Two γ chain variants have been identified which differ not only in mol wt as shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) but also in their carboxyterminal amino acid sequence.[7-9] Most plasma fibrinogen γ chains and all of the γ chains found in platelet fibrinogen are of mol wt 50,000 (γ50).[12,13] In contrast, γ chains of mol wt 57,500 (γ57.5), also known as γ′ chains,[2] constitute ~5% of plasma fibrinogen γ chains[10] and are absent from platelet fibrinogen preparations.[12,13] The increase in mol wt of γ57.5 chains is due to an additional carboxyterminal amino acid sequence beginning four residues prior to the normal γ chain termination.[9] An intermediate length γ chain variant, mol wt 55,000, has also been described and constitutes ~1.5% of total plasma fibrinogen γ chains.[10,11]

The carboxyterminal region of the fibrinogen γ chain is rich in functional sites, for example, for calcium binding,[14] factor XIIIa-catalyzed fibrin crosslinking,[15] and platelet–fibrinogen interactions.[16-20] Because structural variations may influence these functional properties, we compared the ability of fibrinogen possessing only γ50 chains ($F^{\gamma 50}$) with fibrinogen possessing equal amounts of γ50 and γ57.5 chains ($F^{\gamma 50,57.5}$) to bind to platelets and to support ADP-induced aggregation.

MATERIALS AND METHODS

Fibrinogen fractionation. Fibrinogen (Grade L, Kabi AB, Stockholm, Sweden) was chromatographed on a diethylaminoethanol (DEAE) Sephacel column (Pharmacia Fine Chemicals, Piscataway, NJ) using a combined pH and ionic strength gradient.[10,21] Fractions containing fibrinogen with only γ50 chains or with both γ50 and γ57.5 chains were pooled, concentrated by precipitation at 50% saturated ammonium sulfate, and dialyzed against 0.15 mol/L of sodium chloride, 0.05 mol/L of Tris-hydrochloric acid buffer, pH 7.6 containing 10 U/mL aprotinin (Trasylol, Mobay Chemical Co, NY), 0.02 mol/L of ϵ-aminocaproic acid (EACA), and 0.02% sodium azide.

SDS-PAGE. Labeled and unlabeled $F^{\gamma 50}$ and $F^{\gamma 50,57.5}$ were reduced and examined by SDS-PAGE[22] using 7% gels. Fibrinogen associated with platelets following platelet stimulation with 10 μmol/L of ADP was analyzed by SDS-PAGE after gel-filtered platelets (GFP) were centrifuged through silicone oil and after solubilizing the platelet pellets in buffer containing one part 3.3% SDS, 6 mmol/L of *N*-ethylmaleimide, and one part 1% SDS, 12.5 mmol/L Tris-hydrochloric acid, 20% glycerol, 0.05% bromophenol blue, pH 6.8. All gels were stained with Coomassie Brilliant Blue and were dried. Platelet-bound radiolabeled fibrinogen was visualized on autoradiograms prepared using Kodak X-OMAT AR film (Eastman Kodak Co, Rochester, NY).

Removal of von Willebrand factor. Prior to chromatography, the fibrinogen preparation at a concentration of 1 mg/mL contained .15 U/mL of von Willebrand factor (vWF) by Laurell electroimmunoassay,[23] whereas $F^{\gamma 50}$ contained no detectable vWF, and $F^{\gamma 50,57.5}$ contained 0.10 U/mL. For some experiments, the vWF was removed by immunoadsorption. Protein A-Sepharose (Pharmacia) was swollen and incubated at 25 °C for 30 minutes with anti-vWF antiserum (Calbiochem, San Diego) in a ratio of 180 mg Protein

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A-Sepharose to .3 mL of antiserum. Following centrifugation, the Sepharose was washed with buffer (.1 mmol/L of Tris-hydrochloric acid, pH 8.3 containing 10% NP40, 10% deoxycholate, 5 mg/mL of ovalbumin and .2 mol/L of disodium EDTA). An aliquot of .2 mL of washed, suspended Sepharose was then added to 0.8 mL of fibrinogen sample and incubated at 25 °C for 30 minutes, and the supernatant was removed after centrifugation. The buffer was changed to .15 mol/L of sodium chloride, .05 mol/L of Tris-hydrochloric acid, pH 7.6, containing .02 mol/L of EACA, 10 U/mL of aprotinin and .02% sodium azide by chromatography on a column of Sepharose G-100 (Pharmacia). The final preparation contained no detectable vWF by Laurell electroimmunoassay.[23] No change in the migration of bands as determined by SDS-PAGE was noted following this procedure.

Fibrinogen characterization. The γ chain composition of $F^{\gamma 50}$ and $F^{\gamma 50,57.5}$ was confirmed by SDS-PAGE. The γ chains of $F^{\gamma 50}$ migrated as a single band with mol wt 50,000, whereas the γ chains of $F^{\gamma 50,57.5}$ were equally distributed among bands of mol wt 50,000 and mol wt 57,500 (Fig 1). The Aα chain degradation of both $F^{\gamma 50}$ and $F^{\gamma 50,57.5}$ was comparable, and as shown previously,[24] insufficient to influence fibrinogen binding or platelet aggregation.

Fibrinogen iodination. Purified $F^{\gamma 50}$ and $F^{\gamma 50,57.5}$ were labeled with ^{125}I using chloramine T.[25] Following iodination, 99% of the radioactivity associated with fibrinogen preparations precipitated in 10% trichloroacetic acid, and 94% to 98% was clottable by purified human thrombin (1 U/mL) (a generous gift from Dr John J. Fenton, NY State Department of Health, Albany, NY).

Platelet preparation. Blood was collected from volunteers after obtaining informed consent according to the Declaration of Helsinki. It was anticoagulated with 0.1 vol 3.2% sodium citrate in the presence of 0.05 vol 1 mmol/L of acetyl salicylic acid. In some studies, aspirin was omitted. Unless specified, experiments were performed using aspirin-treated platelets to prevent the release of platelet fibrinogen lacking elongated γ chains.[13] Platelet-rich plasma was obtained by centrifugation (280 g, 15 minutes). GFP were prepared as described previously.[25] EDTA-treated and control, Ca-EDTA-treated GFP were prepared as described by Zucker and Grant.[26]

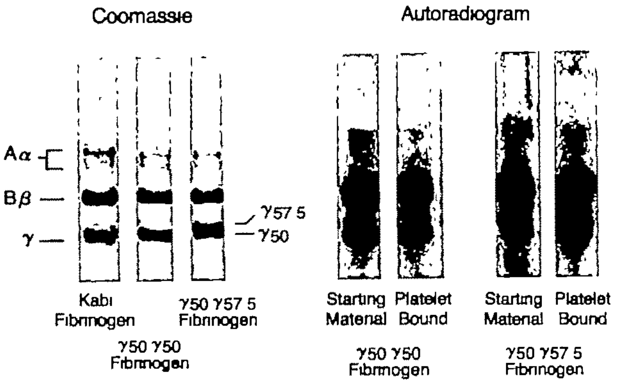

Fig 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of fibrinogen preparations before and after binding to platelets. Electrophoresis was performed in 7% SDS-polyacrylamide gels toward the anode (bottom), following which the gels were stained for protein in Coomassie Blue (left) or processed for autoradiography (right). Protein loading was 10μg for Coomassie-stained gels and 50,000 cpm per lane for autoradiograms. The sample of $F^{\gamma 50,57.5}$ shows the characteristic γ chain doublet. The autoradiograms show the labeled fibrinogen preparation before addition to platelets and that bound to platelets following stimulation with 10 μmol/L ADP.

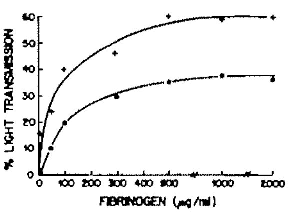

Fig 2. Extent of ADP-induced aggregation of aspirin-treated human platelets at increasing concentrations of homodimer $F^{\gamma 50}$ (+) and heterodimer $F^{\gamma 50,57.5}$ (●).

Platelet aggregation. Platelet aggregation studies were performed using a dual-channel aggregometer (Chrono-Log Corp, Havertown, Pa). GFP (0.45 mL) were stimulated with 10 μmol/L of ADP (Sigma Chemical Co, St Louis) in the presence of either 50 μL $F^{\gamma 50}$ or $F^{\gamma 50,57.5}$ diluted with 0.15 mol/L of sodium chloride to achieve fibrinogen concentrations ranging from 0.01 to 2.0 mg/mL in the final platelet suspensions. The buffer used for dialysis of the fractionated fibrinogen preparations had no effect on the slope or extent of platelet aggregation.

Fibrinogen binding. Platelets stimulated with 10 μmol/L of ADP were incubated with increasing concentrations of radiolabeled fibrinogen for two minutes, after which platelet-bound fibrinogen was separated from free fibrinogen by centrifugation of platelets through silicone oil.[25] The results were analyzed using a Hewlett Packard (Corvallis, OR) HP-85 curve-fitting program according to the method of Scatchard[27] and as recommended by Klotz.[28] The interaction between ADP-treated GFP and radiolabeled fibrinogen was also evaluated at intervals during the first 60 minutes, and fibrinogen dissociation was measured after addition of EDTA (10 mmol/L final concentration) or excess unlabeled $F^{\gamma 50}$ (10 mg/mL).

Displacement of platelet-bound radiolabeled fibrinogen by unlabeled $F^{\gamma 50}$ was studied as follows. ADP-stimulated platelets were first incubated with radiolabeled fibrinogen. After two minutes, a tenfold excess of $F^{\gamma 50}$ was added, and the amount of radioactivity remaining with platelets after 2, 15 and 30 minutes was quantified. Competition of unlabeled fibrinogen with radiolabeled $F^{\gamma 50}$ for platelet receptors was studied by measuring the binding of trace amounts of radiolabeled $F^{\gamma 50}$ to ADP-stimulated platelets in the presence of 0.05, 0.15, and 0.50 mg/mL of unlabeled $F^{\gamma 50}$ and $F^{\gamma 50,57.5}$. In other studies, platelets were incubated for two minutes with near-saturating concentrations of unlabeled $F^{\gamma 50}$ or $F^{\gamma 50,57.5}$ (0.8 to 1.0 mg/mL), and the binding of ^{125}I-$F^{\gamma 50}$, added subsequently to achieve a final concentration of 1.0 mg/mL, was quantified.

The platelet membrane binding sites for $F^{\gamma 50}$ and $F^{\gamma 50,57.5}$ were investigated by incubating GFP (five minutes, 22 °C) with a monoclonal antibody specific for the GPIIb/IIIa complex (10E5, 20 μg/mL final concentration) (a generous gift from Dr Barry Coller, SUNY, Stony Brook).[29,30] Binding of radiolabeled $F^{\gamma 50}$ and $F^{\gamma 50,57.5}$ was evaluated two minutes after platelet stimulation with 20 μmol/L

Table 1. ADP-Induced Platelet Aggregation Supported by Increasing Concentrations of $F^{\gamma 50}$ and $F^{\gamma 50,57.5}$

Fibrinogen (μg/mL)	Platelet Aggregation (Initial Slope)* in the Presence of: $F^{\gamma 50}$	$F^{\gamma 50,57.5}$
10	30 ± 10	15 ± 8
50	45 ± 8	20 ± 6
100	72 ± 11	34 ± 7
300	75 ± 9	32 ± 6
1,000	76 ± 7	36 ± 6

*Light transmission units per minute, mean ± SD, n = 4.

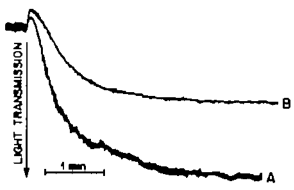

Fig 3. A representative aggregometer tracing of platelets stimulated with 10 μmol/L of ADP in the presence of 0.2 mg/mL of $F^{\gamma 50}$ (A) and $F^{\gamma 50,57.5}$ (B).

of ADP. Platelets incubated with 10 mmol/L of EDTA for 15 minutes at 37 °C and pH 8.1 were also evaluated for their ability to bind $F^{\gamma 50}$ and $F^{\gamma 50,57.5}$ following recalcification and stimulation with 10 μmol/L of ADP. This EDTA treatment causes an irreversible loss of platelet aggregability[26] that is related to alterations of the GPIIb/IIIa complex.[31] Platelets incubated with the combination of calcium (10 mmol/L) and EDTA (10 mmol/L) served as controls.

RESULTS

Aggregation cofactor activity. The extent of ADP-induced primary aggregation supported by $F^{\gamma 50,57.5}$ was ~50% that of $F^{\gamma 50}$ at all fibrinogen concentrations tested (0.01 to 2.0 mg/mL) (Fig 2). The rate of platelet aggregation was similarly diminished (Table 1). A representative aggregometer tracing of GFP stimulated with 10 μmol/L of ADP in the presence of 0.2 mg/mL of each fibrinogen is shown in Fig 3 to illustrate the markedly decreased aggregation of ADP-treated platelets in the presence of $F^{\gamma 50,57.5}$.

Binding to ADP-treated GFP. Both fibrinogens bound to platelets in a specific and saturable manner (Fig 4), but 50% less $F^{\gamma 50,57.5}$ than $F^{\gamma 50}$ bound at saturation. Platelet receptors that remained unoccupied in the presence of $F^{\gamma 50,57.5}$, however, were accessible to $F^{\gamma 50}$ added subsequently (Table 2). Equilibrium binding was attained one minute after incubation of ADP-treated GFP with the radiolabeled fibrinogen preparations (Table 3). Virtually all of both labeled fibrinogens could be displaced from ADP-treated platelets by excess unlabeled $F^{\gamma 50}$ one minute after binding had occurred (Fig 5, Table 3). Approximately half of each radiolabeled fibrinogen remained associated with platelets in the presence of 10 mg/mL of unlabeled $F^{\gamma 50}$ or 10 mmol/L of EDTA after 60 minutes of binding.

Both unlabeled fibrinogen preparations competed with trace amounts of labeled $F^{\gamma 50}$ for platelet receptors (Table 4), although $F^{\gamma 50,57.5}$ competed less effectively. Approximately 1.5-fold more labeled $F^{\gamma 50}$ bound to platelets in the presence of 0.05 mg/mL of $F^{\gamma 50,57.5}$ than in the presence of $F^{\gamma 50}$. This ratio increased to 2.0 at concentrations of unlabeled fibrinogens (0.5 mg/mL) that approached saturation of the platelet receptors. These observations are consistent with the overall 50% decrease in $F^{\gamma 50,57.5}$ binding to stimulated platelets as compared to $F^{\gamma 50}$ binding. Both fibrinogens bound to the same platelet receptor, the GPIIb/IIIa complex, as demonstrated by the complete inhibition of fibrinogen binding after preincubating platelets with an anti-GPIIb/IIIa monoclonal antibody (10E5) (Table 5). In addition, neither fibrinogen bound to platelets that had lost their ability to aggregate following prolonged exposure to EDTA[26,31] (Table 5).

Scatchard analyses of fibrinogen binding to ADP-treated platelets yielded upwardly concave plots (Fig 6). The apparent dissociation constants for high-affinity and low-affinity binding of the two fibrinogen preparations derived according to the methods of Rosenthal[32] and Feldman[33] were not significantly different: 0.124 ± 0.06 μmol/L and 0.105 ± 0.05 μmol/L, respectively, for high-affinity $F^{\gamma 50}$ and $F^{\gamma 50,57.5}$ binding to platelets, and 2.08 ± 1.0 μmol/L and 1.98 ± 0.98 μmol/L for low-affinity binding. The amount of $F^{\gamma 50,57.5}$ bound to platelets at saturation appeared to be 50% less than the amount of $F^{\gamma 50}$ bound. This was confirmed by plotting fibrinogen binding against the log of the fibrinogen concentration, as recommended by Klotz[28] (Fig 7). ADP-treated

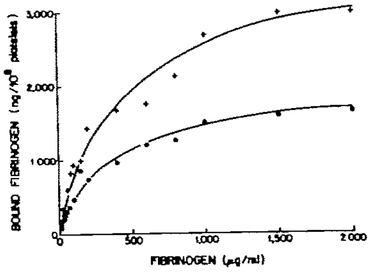

Fig 4. Binding of $F^{\gamma 50}$ (+) and $F^{\gamma 50,57.5}$ (●) to aspirin-treated platelets stimulated with 10 μmol/L of ADP as a function of increasing fibrinogen concentrations.

Table 2. Binding of ^{125}I-$F^{\gamma 50}$ or ^{125}I-$F^{\gamma 50,57.5}$ to ADP-Treated Platelets Preincubated With Near-Saturating Concentrations of Unlabeled $F^{\gamma 50}$ and $F^{\gamma 50,57.5}$

Preincubation Media	Fibrinogen Bound* $F^{\gamma 50}$	Fibrinogen Bound* $F^{\gamma 50,57.5}$
Isotonic saline	31,176 ± 12,365	10,235 ± 4,124
$F^{\gamma 50,57.5}$	19,911 ± 8,147	605 ± 312
$F^{\gamma 50}$	544 ± 611	282 ± 416

Gel-filtered platelets were preincubated in the presence of saline, 1.0 mg/mL of unlabeled $F^{\gamma 50,57.5}$ or $F^{\gamma 50}$ and 10 μmol/L of ADP After two minutes, ^{125}I-labeled $F^{\gamma 50}$, or $F^{\gamma 50,57.5}$ (1.0 mg/mL, final concentration) were added, and their binding was quantified after an additional two minutes by centrifugation of platelets through silicone oil.

*Molecules per 10^8 platelets, mean ± SD, n = 3

Table 3. Fibrinogen Binding to ADP-Stimulated Platelets as a Function of Time

Time (min)	Radiolabeled Fibrinogen Bound (% of Maximum) $F^{\gamma 50}$	Radiolabeled Fibrinogen Bound (% of Maximum) $F^{\gamma 50,57.5}$	Displacement of Bound Fibrinogen (%) by Addition of Unlabeled $F^{\gamma 50}$ (10 mg/mL) $F^{\gamma 50}$	Displacement of Bound Fibrinogen (%) by Addition of Unlabeled $F^{\gamma 50}$ (10 mg/mL) $F^{\gamma 50,57.5}$	Displacement of Bound Fibrinogen (%) by Addition of EDTA (10 mmol/L) $F^{\gamma 50}$	Displacement of Bound Fibrinogen (%) by Addition of EDTA (10 mmol/L) $F^{\gamma 50,57.5}$
1	98 ± 2	100 ± 1	97 ± 5	94 ± 7	98 ± 3	95 ± 4
2	100 ± 4	97 ± 3				
5	100 ± 3	100 ± 2				
15	97 ± 1	98 ± 4				
30	99 ± 4	99 ± 3				
60	100 ± 2	99 ± 5	49 ± 12	60 ± 9	52 ± 15	54 ± 10

Binding experiments of radiolabeled fibrinogen were performed three times, with results indicated as mean ± SD. Fibrinogen displacement was measured five minutes after the addition of unlabeled fibrinogen or EDTA.

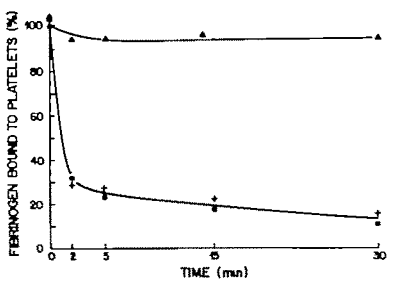

Fig 5. Dissociation of radiolabeled F$^{\gamma50}$ (+) and F$^{\gamma50,57.5}$ (●) from ADP-treated platelets in the presence of 0.15 mol/L of NaCl (upper curve) or 10 mg/mL of F$^{\gamma50}$ (lower curve).

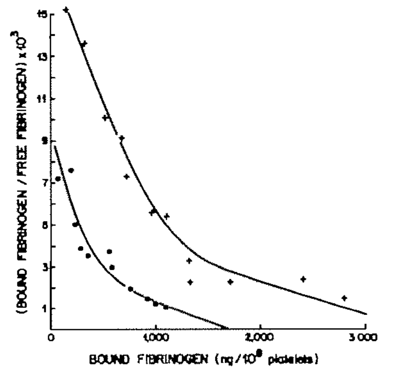

Fig 6. Scatchard plot of F$^{\gamma50}$ (+) and F$^{\gamma50,57.5}$ (●) binding to platelets stimulated with 10 µmol/L of ADP.

platelets bound 53,741 ± 15,653 (mean ± SD) molecules of F$^{\gamma50}$ and 28,563 ± 13,419 molecules of F$^{\gamma50,57.5}$ at saturation. Similar results were obtained with non–aspirin-treated platelets (Table 6), although the overall fibrinogen binding was increased as reported by others.[34]

Although vWF was present in unfractionated fibrinogen and F$^{\gamma50,57.5}$, and it can bind to stimulated platelets,[35,36] little binds in the presence of fibrinogen.[37,38] To confirm this, studies were performed using fibrinogen preparations free of vWF (data not shown). ADP-induced platelet aggregation was not measurably affected by the presence or absence of vWF, and the binding of labeled F$^{\gamma50}$ or F$^{\gamma50,57.5}$ with contaminating vWF could be completely inhibited by a 100-fold excess of unlabeled, vWF-free F$^{\gamma50}$ or F$^{\gamma50,57.5}$, respectively. Moreover, isotope dilution experiments using labeled F$^{\gamma50}$ and F$^{\gamma50,57.5}$ containing trace amounts of contaminating vWF and unlabeled F$^{\gamma50}$ and F$^{\gamma50,57.5}$ free of vWF showed that platelet binding of the labeled and unlabeled fibrinogens was indistinguishable.

Table 4. Binding of Radiolabeled F$^{\gamma50}$ to ADP-Stimulated Platelets in the Presence of Unlabeled Fibrinogens

Unlabeled Fibrinogen (mg/mL)	Radiolabeled F$^{\gamma50}$ Binding (%) in the Presence of Unlabeled Fibrinogen		
	F$^{\gamma50}$ (A)	F$^{\gamma50,57.5}$ (B)	Ratio (B/A)
0.05	30 ± 3	46 ± 4	1.5
0.15	24 ± 1	40 ± 1	1.7
0.50	19 ± 2	38 ± 1	2.0

Binding of radiolabeled fibrinogen was compared to binding in the absence of unlabeled fibrinogen, data are recorded as mean ± SD of five experiments.

Table 5. Effect of Monoclonal Antibody 10E5, EDTA, or Ca-EDTA on the Binding of F$^{\gamma50}$ and F$^{\gamma50,57.5}$ to ADP-Stimulated Platelets

Platelets Incubated With	Fibrinogen Binding* (% of Control)	
	F$^{\gamma50}$	F$^{\gamma50,57.5}$
10E5 (20 µg/mL)	0 ± 2	0 ± 3
EDTA (10 mmol/L) (37 °C, pH 8.1)	5 ± 9	5 ± 11
Ca-EDTA (37 °C, pH 8.1)	98 ± 10	105 ± 11

*Results expressed as mean ± SD of three experiments.

SDS-PAGE of platelet-bound fibrinogens. Because F$^{\gamma50,57.5}$ consists of approximately equal amounts of $\gamma50$ and $\gamma57.5$ chains, studies were performed to examine the mol wt distribution of the γ chains of platelet-associated F$^{\gamma50,57.5}$. As shown in Fig 1, the γ chain distribution of platelet-associated F$^{\gamma50,57.5}$ was the same as that of the F$^{\gamma50,57.5}$ starting material. Electrophoresis of platelet-bound F$^{\gamma50}$ and the F$^{\gamma50}$ starting material served as controls.

DISCUSSION

The predominant γ chain species of normal human fibrinogen has a relative mol wt of ~50,000 ($\gamma50$), whereas higher mol wt variants have been described recently with mol wt of 55,000 ($\gamma55$) and 57,500 ($\gamma57.5$).[7-11] The increase in mol wt of the $\gamma57.5$ chain is due to an elongated carboxyterminal amino acid sequence in which the carboxyterminal four amino acids of the $\gamma50$ chain are replaced by a negatively charged 20 amino acid sequence.[9] Because the γ chains of human fibrinogen appear to possess the major determinants supporting platelet–fibrinogen interactions,[16-19] studies were conducted to compare the ability of a fibrinogen preparation containing only mol wt 50,000 γ chains (F$^{\gamma50}$) and a fibrinogen preparation possessing approximately equal amounts of γ chains of mol wt 50,000 and mol wt 57,500 (F$^{\gamma50,57.5}$) to bind to platelets and support ADP-induced aggregation. The chromatographic elution profile of these fibrinogen preparations and of their constituent chains indicate that F$^{\gamma50,57.5}$ is a heterodimer composed of molecules with one $\gamma50$ and one $\gamma57.5$ chain.[10,11]

Compared to F$^{\gamma50}$, F$^{\gamma50,57.5}$ bound to half as many platelet receptors at saturation (0.8 to 1.0 mg/mL), and supported ADP-induced platelet aggregation less well. These observations are consistent with a recent study by Harfenist et al[39]

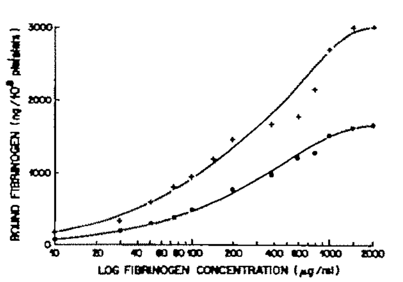

Fig 7. Analysis of F$^{\gamma50}$ (+) and F$^{\gamma50,57.5}$ (●) binding as recommended by Klotz.[28]

Table 6. Comparison of $F^{\gamma 50}$ and $F^{\gamma 50,57.5}$ Binding to Aspirin-Treated and Non-Aspirin-Treated Platelets

	Fibrinogen Binding			
	Non-Aspirin-Treated Platelets		Aspirin-Treated Platelets	
Fibrinogen	K_d (μmol/L)*	Molecules per 10^8 Platelets	K_d (μmol/L)*	Molecules per 10^8 Platelets
$F^{\gamma 50,57.5}$	0.12 ± 0.07/2.35 ± 1.4	38,080 ± 17,506	0.11 ± 0.05/1.98 ± 0.98	28,563 ± 13,419
$F^{\gamma 50}$	0.15 ± 0.05/2.52 ± 1.2	72,141 ± 21,177	0.12 ± 0.06/2.08 ± 1.0	53,741 ± 15,653

*High-affinity binding/lower affinity binding.

All values represent the mean ± SD of four separate experiments.

indicating that fibrinogen which possessed approximately equal amounts of mol wt 50,000 and elongated mol wt 57,500 γ chains was only 50% as effective in supporting ADP-induced platelet aggregation at low concentrations (64 μg/mL) and bound less well to platelets than fibrinogen which contained two γ chains of mol wt 50,000.

The present study extends these observations. A direct correlation has been established between the decreased platelet aggregation support of $F^{\gamma 50,57.5}$ and its decreased binding to exposed platelet membrane receptors over a range of fibrinogen concentrations from 0.01 to 2.0 mg/mL. Moreover, the data suggest that platelet receptors remaining unoccupied in the presence of saturating concentrations of $F^{\gamma 50,57.5}$ are accessible to $F^{\gamma 50}$ fibrinogen.

Despite differences in the extent of fibrinogen receptor saturation, both $F^{\gamma 50}$ and $F^{\gamma 50,57.5}$ appear to bind to the same platelet receptor, the glycoprotein IIb/IIIa complex. The binding of both was completely inhibited by a monoclonal antibody (10E5) specific for this complex,[29,30] and was markedly reduced after platelet incubation with EDTA at an alkaline pH, a process that appears to dissociate the GPIIb/IIIa complex.[31] In addition, both $F^{\gamma 50}$ and $F^{\gamma 50,57.5}$ were shown to bind irreversibly to platelets after 60 minutes.[40]

Both fibrinogens also demonstrated similar binding kinetics. The dissociation constants calculated for high-affinity and low-affinity binding of $F^{\gamma 50}$ and $F^{\gamma 50,57.5}$ were not significantly different. High-affinity binding constants, however, are generally too variable (0.06 to .18 μmol/L) to detect small (twofold or threefold) differences in affinity with certainty. Although these differences could contribute to decreased $F^{\gamma 50,57.5}$ binding, the observation that $F^{\gamma 50,57.5}$ binding approached saturation and failed to increase even at concentrations exceeding saturating doses (> 1.0 mg/mL) suggests that differences in affinity alone are unlikely to be responsible.

High-affinity platelet recognition sites have been localized to the carboxyterminal pentadecapeptide of the fibrinogen γ chain, $\gamma^{398-411}$.[17-19] This segment is composed of highly hydrophilic amino acids, with the exception of valine, and lacks any predominant secondary structure.[18] It possesses the donor and acceptor sites for factor $XIII_a$ catalyzed cross-linking,[15] and is susceptible to proteolysis by plasmin.[41] It has also been shown to inhibit fibrinogen binding to stimulated platelets and, when covalently linked to albumin at sufficiently high density, supports platelet aggregation.[42] Although the smallest γ chain peptide possessing platelet recognition activity is the pentapeptide 407–411,[18] optimum activity apparently requires either the dodecapeptide ($\gamma^{400-411}$)[18] or the decapeptide ($\gamma^{402-411}$).[35]

Several amino acids have been implicated as contributing directly to platelet recognition. Modification of lysine 406, for example, with acetyl anhydride can abolish the inhibitory effect of dodecapeptide $\gamma^{400-411}$ on fibrinogen binding. It has been proposed that this lysine residue participates in the formation of a salt bridge with valine 411 or asparatate 410, which may stabilize a γ chain loop fitting the platelet fibrinogen receptor.[18] Controversy persists regarding the importance of histidine 400 and 401.[18,25]

Thus, most of the amino acids that have been specifically implicated as contributing to platelet recognition, with the possible exception of valine 411, are located beyond the $\gamma^{408-411}$ sequence replaced by the hydrophobic 20 amino acid tail in $F^{\gamma 50,57.5}$. The data, however, demonstrate that the interaction of $F^{\gamma 50,57.5}$ with platelets is markedly decreased. It is possible that loss of valine 411 destabilizes the fibrinogen binding site sufficiently to decrease interaction via the $\gamma^{57.5}$ chain, whereas the hydrophobic extension of the γ chain provides sufficient steric hindrance to prevent saturation of platelet receptors.

The function of elongated γ chains is as yet unknown. Their low concentration in plasma and their decreased support of platelet aggregation may suggest a teleologic selectivity for a more functional γ chain. Fibrinogen secreted from platelets, for example, has been reported to lack elongated γ 57.5 chains,[12,13] and to be more effective than plasma fibrinogen in supporting ADP-induced and epinephrine-induced aggregation.[43] The low concentrations of elongated γ chains present in plasma, however, are unlikely to exert a significant effect on platelet aggregation in vivo.

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Immunoglobulin Gene Rearrangements and Expression in Diffuse Histiocytic Lymphomas Reveal Cellular Lineage, Molecular Defects, and Sites of Chromosomal Translocation

By Katherine A. Siminovitch, Jane P. Jensen, Alan L. Epstein, and Stanley J. Korsmeyer

We have examined the immunoglobulin gene configurations in cell lines from eight patients with diffuse histiocytic lymphoma in order to establish the cellular lineage and stage of differentiation of these lymphomas. The presence of heavy and light chain gene rearrangements as well as heavy chain class switching in seven cells placed these tumors within the B cell lineage. In contrast, one cell (SU-DHL-1), which lacks B cell-restricted surface antigens, retained germline heavy and light chain loci, indicating that it may represent a true histiocyte or uncommitted cell. Truncated RNAs for both the heavy and light chain immunoglobulins were responsible for the lack of surface immunoglobulin in the SU-DHL-2 cell line. Another cell line (SU-DHL-6), which possesses a t(14;18)(q32;q21) translocation, demonstrated an unexpected recombination within its heavy chain gene locus that may be the interchromosomal breakpoint.

THE DIFFUSE histiocytic lymphomas (DHL), as originally defined by the Rappaport classification system, represent a morphologically and immunologically heterogeneous subgroup of malignant lymphomas.[1-3] They consist of a diffuse proliferation of relatively large mononuclear cells which tend to have vesicular nuclei and prominent nucleoli. Because of the marked heterogeneity of these lymphomas, their exact cellular origin was uncertain. Cell surface immunologic markers contributed enormously to clarifying this situation, revealing that many of the lymphomas were of clear B cell or T cell origin.[4-7] Due to these insights, the NCI Working Formulation proposed to classify such cells as intermediate or high-grade diffuse large cell lymphomas.[8] Despite these advances, the exact cellular origin and stage of differentiation of many of these DHLs remained controversial. Many lack surface immunoglobulin (Ig) as well as definitive T-cell markers and have been called "null." It has been proposed that some of these may be "true histiocytes." Furthermore, several of these lymphomas which displayed mature B cell surface antigens failed to produce intact heavy (H) plus light (L) chain surface Ig and must possess molecular genetic defects accounting for this absence of Ig.[7] Finally, several DHL cell lines have been noted to have chromosomal translocations at 14q32, also the site of the Ig H chain gene locus.[9-11] Thus, such cells might display unexpected DNA rearrangements of their H chain gene locus and provide the opportunity to characterize a chromosomal breakpoint.

Because of these unresolved issues, we examined the Ig gene configurations and their expression within established cell lines from eight cases of DHL. The utility of Ig gene rearrangements as B cell-associated clonal markers is well-established. Although all mature B cells possess the mandatory rearrangement of H and L chain genes, other hematopoietic lineages tend to retain germline Ig genes. Specifically, the simultaneous presence of rearranged H plus L chain genes has thus far been an event restricted to B cells.[12-14] Prior studies of human B cell precursors and mature B cell malignancies revealed a development sequence to Ig gene rearrangements in humans in which rearrangements of H chain genes preceded that of L chain genes, and κ rearranged before λ.[12,15-16] In this study, we show that most of these DHLs have rearranged H and L chain Ig genes and are committed to the B cell lineage. However, one cell had completely germline H and L chain genes and may represent a "true histiocyte" or an uncommitted cell. Two cases of B cell origin that lacked surface Ig production were further dissected by examination of their Ig RNA species. Finally, a cell line with a t(14;18)(q32;q21) chromosomal translocation was found to have an unexpected rearrangement within its H chain gene locus that identifies a chromosomal breakpoint.

MATERIALS AND METHODS

Determination of Ig gene configuration. We examined eight cell lines from separate patients with DHL that were previously established and characterized by Epstein et al, Winter et al, and Hecht et al.[4,7,9] High mol wt DNA was extracted from each of these clonal cell lines. These genomic DNAs were digested to completion with the appropriate restriction endonucleases, size-fractionated over agarose gels by electrophoresis, and transferred to nitrocellulose paper. The genomic DNA blots were then hybridized to nick-translated or random-priming incorporated ^{32}P-DNA probes of the Ig gene fragments at specific activities of 200 to 800 cpm per picogram.[12] After washing at the appropriate stringencies, the Ig gene patterns were visualized on autoradiograms. The human Ig gene fragments prepared and used as probes are shown in Figs 1b and 2b. The initial configuration of the H chain genes was assessed with a 2.3-kilobase (kb) *Sau3a* germline fragment of the joining heavy (J_H) gene region hybridized to *Bam*H1-digested or *Eco*R1-digested genomic DNAs.[17] The status of the constant μ (C_μ) and constant γ (C_γ) genes was assessed in *Bam*H1-digested DNA with a 1.3-kb *EcoR*1 germline fragment of C_μ or a 2.3-kb *Hind*III-*Sma*1 germline fragment of $C_{\gamma 1}$, respectively (Fig 1b).[17,18] The rearranged and germline forms of κ genes were discriminated in *Bam*H1-digested DNA probed with a 2.5-kb *Eco*R1 germline fragment of constant κ (C_κ).[19] The λ gene configuration was assessed in *Eco*R1-digested DNA hybridized with a 0.8-kb *Bgl*II-*Eco*R1 germline fragment of the constant λ_1 ($C_{\lambda 1}$) gene capable of cross-hybridizing to all the C_λ genes (Fig 2B).[20]

Analysis of Ig gene expression. Total cellular RNA was prepared from two of the cell lines (SU-DHL-2 and -9) by disrupting the cells with a Polytron (Brinkman Instruments, Westbury, NY) in the presence of 4 mol/L of guanidine thiocyanate. Total RNA was

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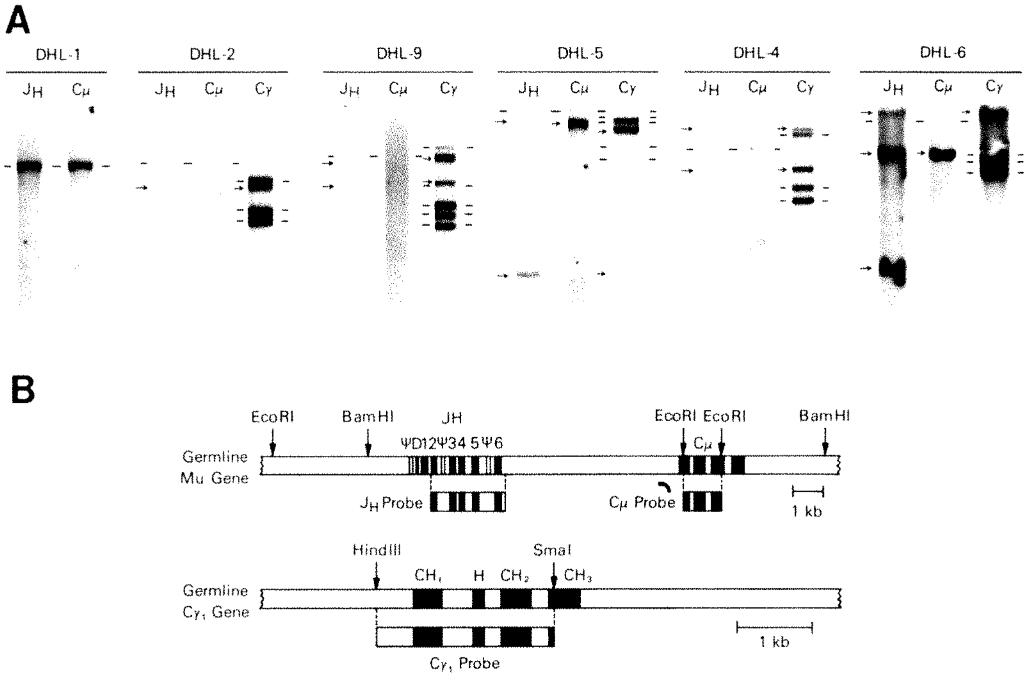

Fig 1. (A) Southern blot analysis of Ig H-chain genes in DHLs. Genomic DNAs are all digested with *BamH1*. The J_H and C_μ probes recognize a germline 17.0-kilobase (kb) *BamH1* fragment indicated by position of the dash marks. The Cγ genes display restriction fragment length polymorphisms (RFLP) for *BamH1* consisting of 25- or 13.5-kb, 12.5-kb, 11.8-kb, 10.0-kb, 9.4-kb or 9.0-kb, and 8.8-kb fragments. Dash marks are shown at the expected position of germline C_γ regions for that particular cell line's RFLP pattern. Several cell lines have deleted C_γ regions on both alleles. H chain class switches produce rearrangements of the *BamH1* fragments possessing the involved C_γ gene. Arrows indicate rearrangements. (B) The J_H probe is a 2.3-kb *Sau3a* germline fragment. The C_μ probe is a 1.3-kb *EcoRI* germline fragment. The $C_{\gamma 1}$ probe is a 2.3-kb *HindIII-SmaI* fragment.

separated from other cellular components by ultracentrifugation through 5.7 mol/L of $CsCl_2$ gradients. Fifteen micrograms of this RNA was size-fractionated over formaldehyde gels and transferred to nitrocellulose paper.[21] These RNA blots were hybridized with the probes noted above.

RESULTS

DHL-1 has germline H and L chain Ig genes. The SU-DHL-1 cell line displayed neither cytoplasmic or surface Ig, HLA-DR, nor the B cell-restricted antigen, B_1 (Table 1).[7,22] Its only B cell-associated marker was BA-1, and this antigen is not solely restricted to the B cell series.[7,23] Examination of Ig gene configurations indicated that the Ig H chain and both L chain gene classes (κ and λ) were retained in their germline form (Figs 1 and 2). H chain genes are known to rearrange very early within the pre-B cell stages of development. In fact, all of the B cell-precursor leukemias that have been examined to date have had H chain gene rearrangements.[12,13,16,23] The earliest identifiable pre-B-cells have HLA-DR and rearrangements of only their H chain genes.[12,23] The lack of HLA-DR and H-chain rearrangement in SU-DHL-1 indicates that it has made no discernible commitment to B cell differentiation. Because T cell-associated antigens (T_3, T_4, T_6, T_8, and T_{11})[7] are also missing, SU-DHL-1 may represent a "true histiocytic" lymphoma.

Abnormal Ig RNA in DHLs with B cell surface markers, Ig gene rearrangements, but no surface Ig. Three of the cell lines, SU-DHL-2, -8, and -9, displayed the B cell-restricted antigen B_1 yet lacked surface Ig. Figure 1 and Table 1 reveal that these cells had rearrangements of their Ig H and L chain genes. The presence of H plus L chain rearrangements, HLA-DR, and B_1, combined with the lack of common acute lymphoblastic leukemia antigen (CALLA), suggest that SU-DHL-2, -8, and -9 are at a mature B cell stage of development.[23] Compatible with a mature B cell stage, all of these cells have undergone deletional H chain class switches.[24] The C_μ genes on both chromosomes have been deleted, and at least one of the J_H regions in each of these lymphomas comigrates with a C_γ region in *BamH*1-digested DNA (Table 1 and Fig 1). Thus, at least one allele in each cell line has correctly switched to a C_γ region yet no γ chain protein is produced. The L-chain gene patterns were similar to those seen previously in other mature κ-producing or λ-producing B cells. SU-DHL-2 and -8, which have rearranged their λ genes, have no remaining germline κ genes, whereas, SU-DHL-9 with a κ rearrangement and some cytoplasmic κ chain detected, has retained germline λ genes. These findings are consistent with the κ before λ order to L chain rearrangements.

Despite the presence of Ig gene configurations characteristic of mature B cells in SU-DHL-2, -8, and -9, they all failed to assemble cell surface H plus L chains. Because adequate numbers of viable SU-DHL-2 and -9 cells were available, we were able to examine their RNA in order to determine if any defects in transcription would account for the lack of surface Ig production. Figure 3 indicates that SU-DHL-2 has abundant C_γ RNA, but that it is abnormally small in size (1,100 base pairs) as compared to the normal-sized γ transcripts of

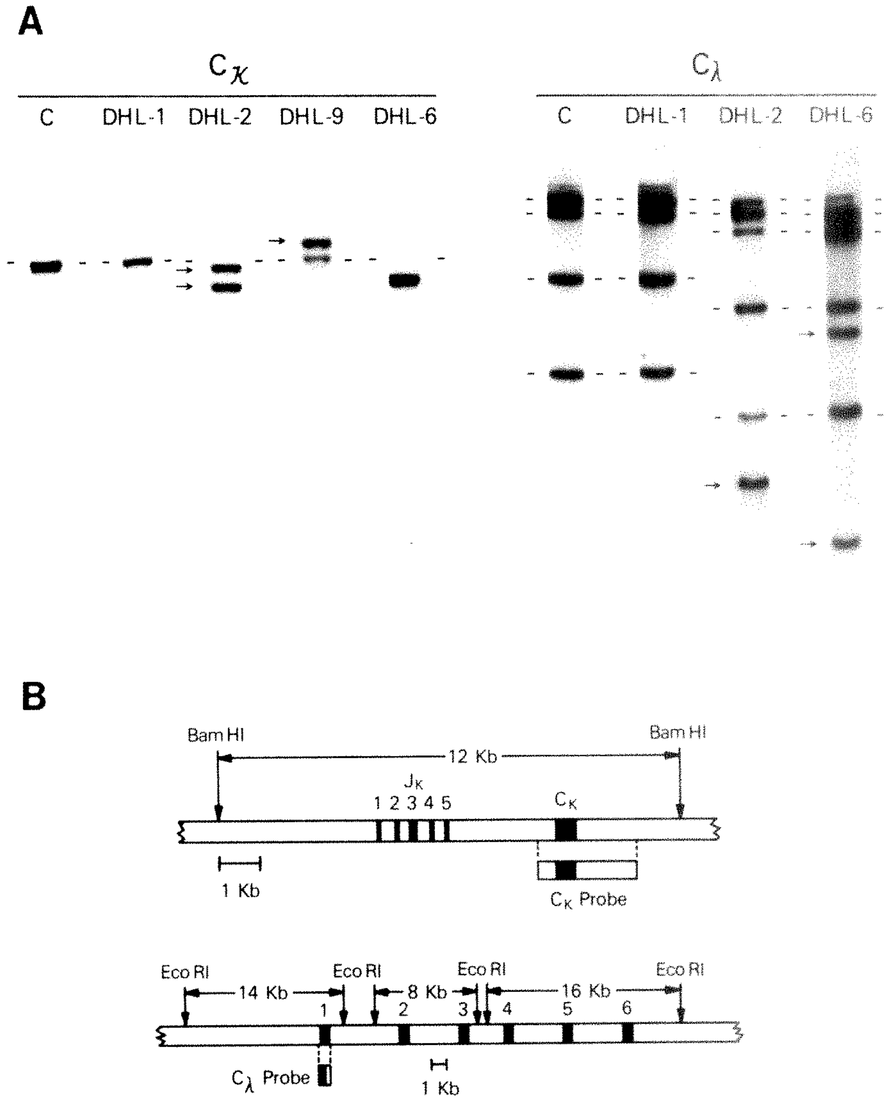

Fig 2. (A) Southern blot analysis of Ig L-chain genes in DHLs. The C_κ probe detects a 12.0-kilobase (kb) *Bam*H1 fragment in its germline form (dash marks) as shown in the source of germline control tissue (C). Rearrangements are indicated by arrows. The C_λ probe displays restriction fragment length polymorphisms in *Eco*R1-digested DNA in which the germline position (dash marks) are 5-kb, 8-kb, 14-kb, 16-kb, and in SU-DHL-2 and -6 also an 18-kb fragment. Rearrangements in SU-DHL-2 and -6 are indicated by arrows. (B) The C_κ probe is a 2.5-kb *Eco*R1 germline fragment. The $C_{\lambda 1}$ probe is a 0.8-kb *Bgl*II-*Eco*R1 germline fragment capable of hybridizing to all of the C_λ-containing *Eco*R1 fragments.

1,900 base pairs (bp). No C_μ-containing or C_α-containing messages were present (data not shown). In addition to the defective H chain RNA, SU-DHL-2 also had a truncated κ RNA of only 750 bp, compared to the normal size of 1,200 bp.

Many mature κ-producing B cells have been noted to generate small amounts of λ gene transcripts (S.J.K., unpublished observations, September 1983) even when their λ genes are in the germline configuration. Similarly, SU-DHL-2 displays detectable λ transcripts that are also smaller than those in neighboring counterpart cells in Fig 3. Thus, SU-DHL-2 has abnormally truncated messages for γ, κ, and λ, indicating the presence of multiple molecular defects and thus accounting for the lack of Ig production. In contrast, SU-DHL-9, which also lacks surface Ig and has undergone a H chain class switch, has an apparently normal-sized γ RNA and a normal-sized κ RNA (Fig 3). Some isolated κ L-chain is found in the cytoplasm of this cell line. Despite the presence of small amounts of normal-sized γ RNA, it fails, however, to produce γ chain, indicating that later defects, perhaps in translation, exist.

B Cell-type DHLs show Ig gene rearrangements compatible with a mature B cell stage. SU-DHL-4, -5, -6, and -7 all revealed rearranged H plus L chain genes (Figs 1 and 2, Table 1). Consistently, all of the cells shown to be κ producers had rearranged their κ genes, and the λ producer (SU-DHL-5) had the obligate λ gene rearrangement. However, an exception to the usual pattern of germline λ L chain genes in κ-producing cells was observed in SU-DHL-6 and -7. Both cells displayed λ rearrangements, despite being κ producers, which is a relatively rare event because κ genes usually rearrange prior to λ (Fig 2).

Both of the IgM-producing lines (SU-DHL-5 and -6) were noted to have one C_μ region which co-occupied the same *Bam*H1 fragment with the rearranged J_H. In the absence of a second C_μ region in these cells, the C_μ-containing alleles are by definition effective rearrangements of $V_H/D_H/J_H$ segments responsible for the production of IgM. The excluded H chain allele in both of these cells has undergone a H chain class switch in which a C_γ region co-occupies a *Bam*H1 fragment with a J_H segment. Thus, IgM-producing B cells can display H chain class switches on their opposite allele.

Table 1. Cell Surface Markers, Immunoglobulin, and Ig Gene Configurations

Case No.	Surface Antigens			Surface Immunoglobulin	Cytoplasmic Immunoglobulin	Immunoglobulin Gene Pattern		
	HLA-DR	B_1	CALLA			Heavy Chain	κ	λ
SU-DHL-1	−	−	−	—	—	Germ	Germ	Germ
SU-DHL-2	+	+	−	—	—	J_H 1 Rearr, 1 Del C_μ 2 Del C_γ 1 Rearr	2 Rearr	1 Rearr
SU-DHL-4	+	+	−	IgG/k	IgG/k	J_H 2 Rearr C_μ 2 Del C_γ 2 Rearr	1 Germ 1 Rearr	Germ
SU-DHL-5	+	+	−	IgM/λ	IgM/λ	J_H 2 Rearr C_μ 1 Rearr, 1 Del C_γ 1 Rearr, Del	2 Del	2 Rearr
SU-DHL-6	+	+	−	IgM/κ	IgM/κ	J_H 3 Rearr C_μ 1 Rearr, 1 Del C_γ 1 Rearr	1 Rearr 1 Del	2 Rearr
SU-DHL-7	+	+	−	—/κ	IgG/κ	J_H 2 Rearr C_μ 2 Del C_γ 2 Rearr	1 Rearr 1 Germ	2 Rearr
SU-DHL-8	+	+	−	—	—/λ	J_H 2 Rearr C_μ 2 Del C_γ 2 Rearr	1 Rearr 1 Del	2 Rearr
SU-DHL-9	+	+	−	—	—/κ	J_H 2 Rearr C_μ 2 Del C_γ 2 Rearr	1 Rearr 1 Germ	Germ

Germ, germline; Rearr, rearranged; Del, deleted; CALLA, common acute lymphoblastic leukemia antigen.
Surface antigens and immunoglobulin as presented in Winter et al.[7]

Furthermore, additional deletional rearrangements have occurred in the H chain gene region of SU-DHL-5. There are four functional human C_γ genes (γ_1, γ_2, γ_3, γ_4) and a nonexpressed pseudogene $\gamma\psi$.[18,25] Some restriction fragment length polymorphism does exist so that the germline C_γ regions in humans occupy *Bam*H1 restriction fragments of 25-kb or 13.5-kb, 12.5-kb, 11.8-kb, 10.0-kb, 9.4-kb or 9.0-kb, and 8.8-kb.[26] Both gene copies of a number of these *Bam*H1 fragments have been deleted in SU-DHL-5 and only a germline copy of the 12.5-kb fragment containing the $C_{\gamma 1}$ gene has been retained (Fig 1). This indicates that C_γ deletional events have occurred on the expressed allele containing the C_μ region as well as on the excluded allele that has undergone a H chain class switch. The presence of a rearranged C_γ gene that lacks an associated J_H region should provide an opportunity to identify the unexpected downstream rearrangement that is mediating this complex deletion (Fig 1).

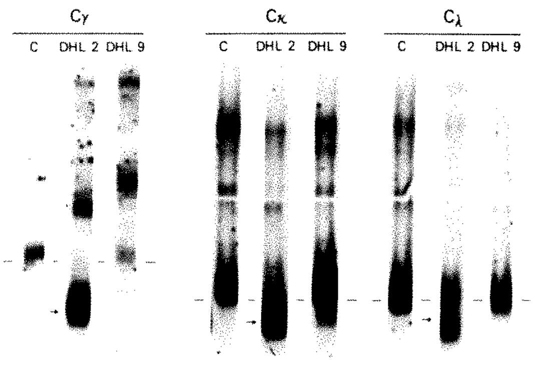

Fig 3. Northern blot analysis of C_γ-, C_κ-, and C_λ-containing transcripts in total RNA of SU-DHL-2 and SU-DHL-9. (C) A control B cell line producing normal-sized RNA as indicated by the dash marks. Truncated transcripts in SU-DHL-2 are noted by arrows. The higher mol wt bands represent precursor forms of the message present in the nuclear RNA portion.

The two cells that produce IgG (DHL-4 and -7) have undergone deletional H chain class switches (Table 1, Fig 1). H chain class switches have occurred on both the expressed and excluded allele so that no C_μ regions are retained. Therefore, the IgG-producing DHLs are at a relatively mature stage of B cell development and have already undergone an H chain class switch. Thus, there is no evidence that these surface IgG-positive B cells have long, alternatively spliced transcripts which generate IgG, as has been proposed to exist for some B cells producing distally located isotypes.[27]

Chromosomal translocations in DHL may involve the Ig gene loci. Two of the cell lines examined (SU-DHL-4 and -6) were known to possess t(14;18)(q32;q21) chromosomal translocations. Previous extensive examinations of the t(8;14)(q24;q32) translocations of Burkitt's lymphoma revealed that the H chain gene locus at 14q32 was itself often mediating these translocation events. To determine whether the H chain gene locus was similarly involved in the rearrangements with 18q21, we examined the H chain genes in SU-DHL-4 and -6 to see if any unanticipated rearrangements had occurred. Specifically, we searched for rearrangements that could not be accounted for by the routine events of $V_H/D_H/J_H$ assembly or H chain class switch. Only the expected rearrangements of J_H and C_γ segments were observed in SU-DHL-4, typical of an IgG producer. A very provocative finding was made in SU-DHL-6, however. This diploid cell line was known to have one normal chromosome 14 and a derivative 14q+ chromosome. However, Southern analysis revealed the presence of three instead of the usual two J_H-containing *Bam* fragments (Fig 1). One of these J_H fragments (10-kb) comigrated with the C_μ region in a *Bam*H1 digestion, and thus corresponds to the effectively rearranged $V_H/D_H/J_H$-C-containing allele responsible for the IgM produced and presumably is located on the normal chromosome 14 in this cell. We proposed that the other two J_H fragments (22-kb and 4.0-kb) were created by a chromosomal breakpoint within the J_H region of the phenotypically excluded allele. Consistent with this hypothesis, the 22-kb *BAM*H1 fragment has an associated C_γ region together with the J_H segment, whereas the 4.0-kb J_H-containing *Bam*H1 fragment has no identifiable Ig gene information at its 3′ side (Fig 1). Subsequently, the 22-kb fragment has recently been shown to possess chromosome 18 information at its 5′ end and to correspond to the derivative 14q+ chromosomal breakpoint.[28] The 4.0-kb allele would thus possess chromosome 18 information at its 3′ end and would correspond to the derivative 18q− chromosomal breakpoint.

DISCUSSION

We have examined the status of Ig genes within DHLs. We have used their Ig genes as markers of cellular lineage, their state of differentiation, Ig production defects, and potential chromosomal translocation sites. The DNA rearrangements of Ig genes have proven of enormous value in assigning a B cell lineage to a number of lymphoid neoplasms of uncertain classification. This includes the non-T form of acute lymphoblastic leukemia,[12,16,23] the lymphoid blast crisis phase of chronic myelogenous leukemia,[13] hairy cell leukemia,[29] and many lymphoma biopsy tissues.[14,30] This is based on the fact that hematopoietic cells which pursue other than a B cell pathway of development usually retain their Ig genes in the germline form. Exceptions do occur for the H chain genes which occasionally rearrange in human T cells (2 of 23) and even myeloid cells.[12,14] However, the L chain genes have been uniformly retained in a germline form in T cells, myeloid, promyelocytic, and monocytic cell lines and leukemias. Thus, the simultaneous detection of rearranged H plus L chain Ig genes serves as a strong marker for B cell lineage commitment. Since the histologic description of DHL, there has been considerable controversy concerning the cellular origin and state of maturation of the markedly heterogeneous affected cells. Many of the DHLs could be classified—by using immunologic surface markers, as T- or B-cell in nature—but others were placed within a "null" or true histiocyte category only by default.

In this study, we have examined the Ig gene configuration and expression in eight well-established and characterized cell lines. We show that most of these lines are at a mature B cell stage, possessing H plus L chain rearrangements, and that they may have already undergone a H chain class switch. Of the four that fail to make surface Ig, one (SU-DHL-1) retains germline H and L chain genes. Furthermore, two monoclonal antibodies (2H9, 1E9) produced by immunizing with SU-DHL-1 reacted with the nuclear membrane of histiocytes and interdigitating reticulum cells in normal lymphoid tissues.[31] Moreover, SU-DHL-1 also displayed the monocytic marker Leu M5 and retained the same phenotypic markers after induction with phorbol ester.[31] As noted above, SU-DHL-1 also lacks definitive B cell or T cell surface antigens. SU-DHL-1, therefore, provides an important cell line model that may be a "true histiocyte." The other three cells lacking surface Ig (SU-DHL-2, -8, and -9) are clearly at a mature B cell stage of development and must possess molecular defects that prevent Ig production. Multiple molecular defects have been identified in SU-DHL-2, which has truncated H and L chain RNAs. This cell line may be an appropriate setting to search for a common molecular mechanism that results in the loss of Ig H and L chain production.

Several other observations concerning the configuration of Ig genes in lymphomas of mature B cell phenotype are noteworthy. Our examination of SU-DHL-4, -5, -6, and -7 indicate that the excluded, nonproductive H chain allele in these cells has undergone a H chain class switch. This is not unexpected in IgG producers in which the same molecular switching mechanism may be operative on both chromosomes. However, SU-DHL-5 and -6 are IgM producers, and their excluded allele has also undergone a H chain class switch to a C_γ region. We have also noted this phenomenon at times in other mature IgM-producing B cell lines and leukemias. This raises important questions concerning the regulation of this event and its purpose for the cell. Furthermore, it provides a very clear marker to distinguish on a Southern blot which H chain allele is indeed responsible for Ig production. The L chain genes in humans appear to rearrange in an ordered sequence in which κ genes precede λ.[12,16] A consequence of this is that λ genes are usually retained in their germline form within κ-producing B cells.[15,32] SU-DHL-6 and -7 are κ producers which show clear exceptions to this general pattern, having λ gene rearrangements. Whether these were present prior to transformation or were acquired in vivo or in vitro is uncertain, but they do indicate that λ rearrangements can at times occur in κ-producing cells. We have recently identified a κ-deleting element (κde) which is rearranged to disrupt the κ locus prior to the time of λ gene rearrangement.[33] SU-DHL-6 has rearranged a copy of this κde on its excluded κ gene allele. The rearrangement of the κde may prove to be related to the progression from κ to λ rearrangements.

Two of the DHL cell lines (SU-DHL-4 and -6) possess chromosomal translocations t(14;18)(q32;q21) usually associated with the nodular (follicular) type of lymphomas.[9,34] However, these diseases may prove to be a continuum because some lymphomas that initially present with nodular histology progress to a diffuse architecture over time.[35] The chromosomal break at 14q32 is the same band in which the human H-chain genes are located.[10,11] However, such a chromosomal band may contain 1,000 to 10,000 kb of information in addition to the Ig locus. Yet, examination of Burkitt's lymphomas revealed that their chromosomal breakpoint at 14q32 was actually situated within the H chain gene locus, with the breakpoint frequently occurring between the J_H and C_μ regions.[36,37] In this study, we have shown that SU-DHL-6 has one normally rearranged H chain allele which accounts for its IgM production. The other allele has a J_H region which unexpectedly falls on two separate *Bam*HI restriction fragments. This observation has allowed us to clone the reciprocal breakpoints of this chromosomal translocation and should enable us to determine the identity of the information being introduced from chromosome 18.[28] The presence of this definable breakpoint within a DHL cell line provides the opportunity to assess the contribution of this translocation to the malignant phenotype of diffuse as well as follicular lymphoma.[38]

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Altered Stem Cell (CFU-S) Function Following Infection of Hematopoietic Cells With a Virus Carrying V-*src*

By David Boettiger and T. Michael Dexter

Long-term murine bone marrow cultures were used to support the growth and development of hematopoietic cells. After hematopoiesis was established, the cultures were infected with a recombinant murine amphotropic virus carrying the avian sarcoma virus *src* gene and the CFU-S kinetics were examined. The CFU-S from the *src*-infected cultures displayed a reduced seeding efficiency in the standard spleen colony assay. The self-renewal capacity of these CFU-S was tested by their ability to reestablish hematopoiesis when serially transplanted on irradiated bone marrow cultures and by serial passage in spleens of irradiated mice. In both tests, cells from the *src*-infected cultures exhibited an enhanced ability to sustain a high level of self-renewal. The other property of stem cells which may be measured is the probability of self-renewal at each cell division which dictates the distribution between stem cells and differentiated type progeny. CFU-S from the *src*-infected cultures had higher average probabilities of self-renewal and therefore reduced differentiation. These differences suggest that expression of *src* had indirectly or directly altered the normal differentiation program of the stem cells.

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ONE OF THE prominent effects of at least some oncogenes is a reduction in the ability of the affected cell to maintain its normal differentiation program. This has been particularly well demonstrated for the *src* oncogene through the use of temperature-sensitive mutants along with specific cell markers available for particular differentiated cell types. These studies have demonstrated that the synthesis of muscle-specific proteins in myotubes,[1,2] the synthesis of cartilage-specific extracellular matrix components by chondroblasts,[3,4] the synthesis of extracellular matrix components by fibroblasts,[5] and the production and maintenance of melanosomes by pigmented retina cells[6] are suppressed by the expression of *v-src* in these cell types. Further experiments in the chondroblast and fibroblast systems have demonstrated that the differences in synthesis of these products is controlled primarily at the transcriptional level.[4] Suppression or inhibition of expression of the mature cell phenotype is not an effect restricted to the *src* oncogene, but has also been demonstrated for the *myb* oncogene in macrophages[7] and for the *erb* oncogenes in erythroid cells.[8] In each of these cases, the affected cell is one which is already committed to particular lineage and which in most cases has initiated the synthesis of the definitive cell type-specific products. Few studies have examined the potential of oncogenes to affect properties of progenitor cell populations. An attempt has been made to infect chondroblast progenitors which are present in day 3 chick limb buds. These experiments demonstrated that the *src* oncogene did not prevent the differentiation of these cells into chondroblasts and may, in fact, have promoted the process of differentiation.[9] This suggests that the effects of oncogenes in the progenitor cell compartments of differentiation may be different than their effects on the definitive cell types.

To investigate further the effect of oncogenes on progenitor cell compartments, we turned to the murine hematopoietic cell system. The chief advantages of this system are the availability of the long-term bone marrow (LTBM) culture for the maintenance of the hematopoietic stem cells and the availability of quantitative assays for both the stem cell and the committed progenitor cell stages of differentiation.[10] The only effects on hematopoietic cells which may be ascribed to *scr* are induction of hemangiomas[11] and transformation of erythroid cells,[12] but leukemias have not been reported. We have recently described the effects of a recombinant murine retrovirus carrying the avian *v-src* gene on murine LTBM cultures.[13,14] There was an overall reduction in the production of nonadherent hematopoietic cells, reflecting a reduction in the granulocyte cell population and a dramatic increase in the proportion of blast cells. This was accompanied by a 25- to 50-fold increase in the level of GM-CFC (the granulocyte-macrophage progenitor cells) and CFU-S (the multipotential stem cells) on a per culture basis. These data suggested that the hematopoietic progenitor cells represented by these two compartments may be altered as a consequence of the infection. Cells from the *src*-infected LTBM cultures could be serially passaged using either the standard GM-CFC assay conditions or the in vivo spleen colony assay for more passages than the controls or than had been previously observed. This implied that the alteration in the progenitor cell population persisted in cells removed from the microenvironment of the *src*-infected cultures. Additional studies on the GM-CFC agar colonies have demonstrated that these colonies are actually initiated by multipotential cells rather than by cells committed to the GM-CFC lineage.[15] The combined data imply that the primary lesion in the hematopoietic cells is intrinsic and appears to be primarily at the stem cell or multipotential cell level.

MATERIALS AND METHODS

LTBM cultures were prepared from young adult B6D2F1 mice and were infected after five to eight weeks with the recombinant murine amphotropic virus[13] using a Moloney leukemia virus helper virus.[10,14] Cells were removed from the nonadherent pool at the time of feeding and were assayed for CFU-S[16] by inoculation into

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B6D2F1 recipients which had been irradiated with 13.5 Gy ^{137}Cs-rays (dose-rate 0.85 Gy/hr). Mice were killed at 11 days, the spleens removed, fixed in Bouin's solution, and the number of spleen colonies counted. All assays included two to three different doses of inoculated cells, and eight to ten mice were used per group; a background group was included with each experiment. The SE was calculated as is given in Tables 1 through 5 or as error bars on Figs 1 through 3 as appropriate. For neuraminidase treatments, the cells were incubated for 30 minutes at 37 °C with 0.2 U/mL of *Clostridium perfringens* neuraminidase (Sigma) prior to inoculation.

To calculate the seeding efficiency (ie, the fraction of potential CFU-S which seed in the spleen and produce a spleen colony), lethally irradiated mice were inoculated intravenously (IV) with 10^4 to 10^6 cells from *src*-infected cultures or from control cultures. Some animals inoculated with low cell doses were kept for counting of spleen colonies at 11 days. Some animals inoculated with higher cell doses were killed at 24 hours; their spleens were removed, and single cell suspensions were prepared. Aliquots of these single cell suspensions were inoculated into secondary lethally irradiated recipients, and spleen cell colonies were counted after 10 to 11 days. The *f* number was calculated by the formula[17]: Number of CFU-S colonies in secondary recipients/number of CFU-S colonies in primary recipients, corrected for the differences in initial inoculum size.

RESULTS

CFC-S from Src-*infected LTBM cultures have an altered spleen seeding efficiency.* The seeding efficiency of CFU-S obtained from the *src*-infected cultures was measured at different times after infection for two separate experiments. The experimental procedure compares the spleen colony formation for cells that had to seed the spleen once to those that have to seed the spleen twice. Table 1 shows a seeding efficiency (*f* number) for CFU-S obtained from control cultures of 0.11 and 0.15 which is in close agreement with previous data obtained for either CFU-S from LTBM cultures or CFU-S from fresh BM (references 17 and 18, and Dexter, unpublished observations). In contrast, the seeding efficiency for the CFU-S from the infected cultures was 0.018 to 0.024, or about fivefold to sixfold less than the controls. The seeding efficiency of CFC-S in the spleen appears to depend on cell surface properties.[19] The difference in the seeding efficiency implies an intrinsic difference between the CFU-S obtained from the *src*-infected cultures and CFU-S from normal LTBM cultures.

The hypothesis that seeding efficiency is determined by properties of the cell surface is supported by the observation that treatment of the CFC-S (the cells which produce CFU-S) with neuraminidase (which removes the sialic acid from cell surface glycoproteins and glycolipid) results in a decrease in the seeding efficiency.[19] To determine whether neuraminidase treatment would have a similar effect on the CFC-S from the *src*-infected cultures, nonadherent cells from control and *src*-infected LTBM cultures were treated with neuraminidase, and the efficiency of spleen colony formation was measured. The results in Table 1 show the relative decrease in colony formation by neuraminidase-treated cells in comparison to untreated cells from the LTBM cultures. Neuraminidase treatment reduced the colony-forming efficiency of the normal CFU-S by approximately sixfold, which confirms the previous results.[1-19] The CFU-S from the *src*-infected cultures showed a reduction of ~3.5-fold following neuraminidase treatment. Although this result is compatible with differences in seeding efficiency being determined by cell surface properties, it does not exclude the participation of additional factors.

The individual spleen colonies produced following the inoculation of cells from the *src*-infected LTBM cultures were morphologically normal and displayed the same distribution of erythroid, granulocyte, and mixed colonies as do spleen colonies derived from cells taken from normal LTBM cultures.

Serial transplantation of CFU-S from Src-*infected cultures.* The serial transplantation of CFU-S from normal BM in vivo in the spleens of lethally irradiated recipients leads to a progressive decline in the number of recoverable CFU-S per input CFU-S until there are insufficient CFU-S in the inoculum to continue the transplantation.[20] To optimize the number of transplant generations, transplantations are separated by about four weeks. In contrast to the results obtained with normal BM CFU-S and those obtained with CFU-S from normal LTBM cultures, CFU-S from the *src*-infected cultures could be passaged more frequently and did not show the usual decline in CFU-S self-renewal.[14]

To examine the self-renewal potential of the CFU-S from the *src*-infected cultures, nonadherent cells were serially passaged in spleens of irradiated recipients at 9- to 20-day intervals. The data for this experiment are given in Table 2.

Table 1. Seeding Efficiency

Experiment	*f* No.	Neuraminidase
Control 23 wk	0.100 ± 0.0200	0.018 ± 0.002
Src 2–23 wk	0.024 ± 0.005	0.007 ± 0.001
Control 11 wk	0.150 ± 0.0700	
Src 2–11 wk	0.018 ± 0.006	

f Number was calculated as the relative recovery of CFU-S from the spleens of primary recipients at one day after inoculation. Neuraminidase was calculated as the relative reduction in efficiency of spleen colony formation after neuraminidase treatment. This value has been corrected for seeding efficiency

Table 2. Serial Transplantation of CFU-S From SRC-Infected Cultures

Transplant Generation	Transplant Interval (d)	CFU-S Transplanted	CFU-S Recovered
1	11	1.90	13.0 ± 1.2
2	11	0.13	15.9 ± 3.7
3	11	0 20	21.8 ± 4.0
4	20	0.44	04 7 ± 3.3
5	11	0.40	05 3 ± 3.2
6	11	0 53	05.0 ± 1 6
7	9	0 50	00.6 ± 1.1

Cells from *src*-infected long-term bone marrow cultures were inoculated IV into syngeneic, irradiated recipients in the standard CFU-S assay. After the indicated transplant interval, mice were sacrificed, spleen colonies were counted, and spleen cell suspensions were made from a pool of two to three mice and diluted and inoculated into the next generation of irradiated recipients The CFU-S transplanted per recipient mouse based on the spleen cell counts and the mean CFU-S recovered per mouse are given. The recovered values give the SEM

Using this data in combination with the seeding efficiency data given in Table 1 and the fact that no increase in CFU-S can be detected during the first three days after inoculation, the growth curve for CFC-S shown in Fig 1 was calculated. The lack of increase in CFU-S during the first three days after inoculation has been demonstrated in previous experiments on normal BM CFU-S[20] and was confirmed for CFU-S from the *src*-infected cultures (data not shown). The seeding efficiency was only determined experimentally for the initial point and not at each serial passage. Figure 1 shows that a relatively constant doubling time for these CFC-S of 23 hours was maintained throughout the 60 days of the experiment. There was no indication of a slowing of the growth curve at the higher passages and hence no suggestion that the passaging could not have been continued for more passages. The ability of the CFC-S from the *src*-infected cultures to be serially passaged in this fashion implies that there is an intrinsic change in these CFC-S which allows them to maintain their capacity for self-renewal under conditions inimical to the maintenance of normal stem cells.

Because the injected cells were taken from cultures producing the *src* virus, it was possible that it was the virus rather than the cells which was responsible for the results in Fig 1. In control experiments, either the *src* virus or cells from *src*-infected cultures, which were irradiated with 15 Gy, were inoculated: no spleen colonies were produced. Because the irradiated cells would still continue to produce virus, the possibility that the spleen colonies produced were due to virus spread and endogenous spleen colony stimulation can be ruled out. It is formally possible that some transformed cells were inoculated as part of the nonadherent cell population taken from the infected LTBM cultures, and that these participated in the rescue of endogenous CFU-S. Although this may explain the initial transplantation, it does not appear to explain the serial passages or the production of secondary colonies from individual spleen colonies (Table 3).

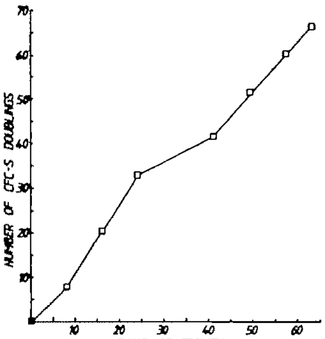

Fig 1. Cumulative growth curve for CFC-S from *src*-infected cultures. The growth curve was calculated from a successive retransplantation of CFU-S from cultures infected with the *src*-containing recombinant virus 24 weeks before the initial transplant. The CFU-S values were corrected for the seeding efficiency, and it was assumed that there was no growth for the first three days after inoculation. The 66.1 cell doublings calculated correspond to an increase of 7.9 × 10^{19}-fold.

Table 3. Calculation of *P* Value by Individual Colony Analysis

Colony	Colonies Analyzed	Mean CFU-S per Colony ± SD	CFC-S*	P†	Tc‡ (h)
Normal LTBM cultures§	106	10.54 ± 23.30	81	0.582	6 9
Normal bone marrow§	362	28.0 ± 37.24	215	0.683	8.6
Src-infected LTBM cultures	28	06 11 ± 7.96	290	0.700	9.3
Src-infected fifth in vivo TP‖	15	07 60 ± 8.75	ND	0.728	10.2

LTBM, long-term bone marrow, ND, not done.

*CFC-S are spleen colony-forming cells calculated from the CFU-S and the *f* number (Table 1) Values are mean CFC-S per spleen colony at 11 days

†*P* values were determined using the formula of Vogel et al[23] and Schofield et al,[20] where m = mean no. of CFU-S per colony; and v = SD/m $v^2 = (2 - 2p)/(2p - 1) + 1/m$ or $p = (2m + mv^2 - 1)/(2mv^2 + 2m - 2)$.

‡Tc is the cell cycle time calculated from the formula of Schofield et al,[20] where c (no of cell cycles) = $\ln m/(\ln 2)(2p - 1)$, and Tc = $192/c$ for the 11-day spleen colonies

§Data from Schofield and Dexter.[21]

‖These cells were derived from the fifth in vivo passage as spleen colonies of cells derived from *src*-infected cultures

Serial transfer of CFU-S in vitro. The self-renewal capacity of the CFU-S from the *src*-infected long-term bone marrow cultures was examined in vitro by serial passage of the non-adherent cells on 4- to 10-week-old normal long-term bone marrow cultures which had been irradiated with 15 to 20 Gy prior to transfer. When normal cultures were given this dose of radiation, endogenous hematopoiesis was completely ablated, but the intact stromal layer could be used as a feeder to support the growth of the inoculated hematopoietic cells.[21] Table 4 shows the results obtained from six serial transfers which were performed at approximately biweekly intervals. The numbers in the boxes indicate the number of CFU-S which were inoculated per flask to initiate each successive transfer. For passages 3 through 6, these numbers were calculated by interpolation and were not measured directly at the time of transfer. The other numbers in Table 4 are the measured number of CFU-S per flask in the nonadherent cell population at different assay times. The cultures were fed at the times given in column 1 using the standard procedure for LTBM cultures (removal of half of the medium and half of the nonadherent cell population). At each transfer, there was both maintenance and growth of the CFU-S population in the culture. The initial burst in CFU-S production, particularly prominent in the first transfer, may have been the result of altered growth factor conditions, since these are known to control CFU-S multiplication in these cultures.[22] The relative decrease in CFC-S proliferation at later times probably resulted from the degeneration of the irradiated adherent monolayer. This degeneration occurred primarily as a result of the initial irradiation of the stromal cells.[14] Although this resulted in some impairment of the ability of the new adherent layers to support the CFC-S, it is worth noting that the passaged CFC-S proliferated following each successive passage. Furthermore, the irradiated stromal layers continued to support the proliferation of CFC-S after the parental cultures had become nonhematopoietic. The ability to maintain sufficient CFC-S self-renewal to permit reestablishment of hematopoiesis through multiple serial passages distinguished the CFC-S in the *src*-infected cultures from CFC-S in control cultures. The CFC-S in the nonadherent cell population of the control LTBM cultures rapidly lose their self-renewal potential when serially trans-

Table 4. In Vitro Passage of CFU-S From *src*-Infected Cultures

Days*	P 1	P 2	P 3	P 4	P 5	P 6
0	$\boxed{2{,}000}$					
8						
11	10,900					
14	—					
20	1,760	$\boxed{176}$				
26	—	504				
34	—	—	$\boxed{350}$			
40	—	—	—			
47	—	—	—	$\boxed{170}$		
49	288	893	816	—		
55	—	—	—	—		
61	—	—	—	—	$\boxed{160}$	
68	—	—	—	—	—	
75	213	45	47	76	156	
82	—	—	—	—	—	$\boxed{11}$
90	—	—	—	—	—	—
96	—	—	—	—	—	—
104	—	—	—	—	—	—
110	150	19	44	198	—	38

*Time of feeding and removal of one-half of the cells.

Numbers in boxes indicate the number of CFU-S initially added per culture of irradiated bone marrow feeder cells (for passages 3 through 6, the starting values were calculated by interpolation) The other values indicate the number of CFU-S per culture taken at the various sampling times. On day 95 after initiation of the serial transfer, the original *src*-infected cultures contained 5 CFU-S per culture and none by day 139.

ferred in vitro, and it is rarely possible to perform even two serial in vitro transfers.[21]

CFC-S: Self-renewal v *Differentiation.* The growth of CFC-S populations is characterized by the production of progeny which retain the properties of the parental CFC-S (self-renewal) as well as of progeny cells with a more limited potential which produce the functional differentiated cells. Thus, the actual increases in the stem cell numbers may be described by two parameters: (a) the average cell cycle time, and (b) the *P* value, or probability that a daughter cell following division of a stem cell is also a stem cell, ie, the probability of self-renewal. In normal homeostasis, it is likely that both *P* value and cell cycle time vary in response to the demands on the system. Unfortunately, neither of these parameters can be satisfactorily measured independently, and available methods depend upon secondary assumptions.

The value of *P* was determined using the assumptions of the stochastic model.[23] The model assumes that at each CFC-S cell division each daughter has the same probability (*P*) of being a CFC-S. This probability can be determined from the analysis of the mean and coefficient of variation SD/mean CFU-S per colony) for the CFU-S content of individual spleen colonies. Individual spleen colonies intiated by CFC-S harvested from *src*-infected cultures and from the fifth in vivo passage of CFC-S from *src*-infected cultures were excised, and each colony was divided and injected into three lethally irradiated recipients to determine the number of CFU-S in each colony. The cells were taken from the long-term cultures at the normal weekly feeding, at which time the normal CFU-S are quiescent.[22] This cell cycle stage difference as well as the time in culture may account for the lower *P* value for CFU-S from control cultures when compared with CFU-S from fresh bone marrow (Table 3). Cells taken directly from the *src*-infected cultures or from the fifth serial in vivo passage of cells from these cultures exhibited a *P* value somewhat higher than that of CFU-S from the uninfected LTBM cultures and slightly above the value obtained for fresh BM. Although CFU-S from normal BM may achieve *P* values similar to that observed for the CFU-S from the *src*-infected cultures, these higher *P* values were not maintained in the LTBM cultures in the absence of *src*.

Previous experiments have demonstrated that increase in CFU-S begins at approximately day 3 in the typical spleen colony assay.[20,24] Hence, it was assumed that eight days' growth of CFU-S occurred in the production of the 11-day spleen colonies. From the *P* value, the measured mean increase in the CFU-S population, and the time of eight days, the average cell cycle time for each population was calculated (Table 3).

Proliferation of CFU-S during spleen colony formation. When CFU-S are inoculated into irradiated recipients, there is an initial lag phase followed by a phase of logarithmic CFU-S growth (usually between four and 12 days after inoculation but dependent on the size of the inoculum). CFU-S from different embryonic and adult tissue sources had characteristic doubling times which ranged from 16 to 25 hours.[24] This parameter appears to measure an intrinsic growth property of the CFU-S, which would include both the cell cycle time and the *P* value. The rate of CFU-S doubling during the logarithmic growth phase was determined for CFU-S taken from the *src*-infected cultures as an alternative measure of CFU-S growth and differentiation.

Nonadherent cells were taken from *src*-infected cultures at different times after infection and inoculated IV into irradiated recipients. At times between five and 12 days after inoculation, pairs of mice were sacrificed, and the CFU-S content of their spleens was determined. The data were plotted to determine the growth rate of the CFU-S during the initial logarithmic growth phase (Fig 2). The three separate growth curves obtained from the *src*-infected cultures show very different growth rates, with doubling times ranging from 15.7 to 79 hours. It was possible that the different input levels of CFU-S in the different experiments were responsible for these differences, although previous experiments had demonstrated that CFU-S doubling times were independent of inoculum size for inoculations up to 10^6 BM cells.[24] However, due to the high CFU-S input of the *Src* 2 (7 week) time point (see table 5), it was necessary to extend the study of the normal CFU-S. When 5×10^6 cells containing 950 CFU-S were inoculated, the doubling time was similar to that of the previous studies, although the plateau level was achieved earlier. This confirmed that it was the intrinsic differences in the cells which was responsible for the differences in the growth rate.

The nonadherent cells used in these determinations were derived from cultures at different times after injection. *Src* 2 (seven weeks) was taken at the peak of the rise in CFU-S in the cultures following a 50- to 100-fold rise in a period of less than two weeks. After this peak, there was a slight decline in the total CFU-S per culture and the establishment of a new equilibrium between the CFU-S and the more differentiated

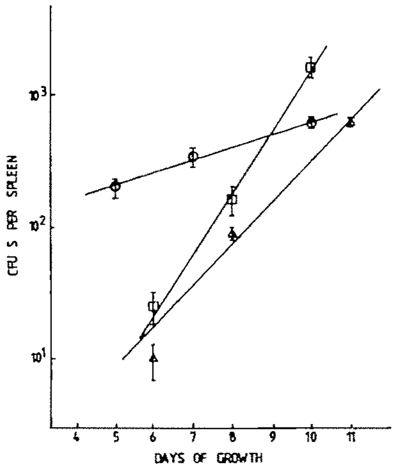

Fig 2. Short-term growth curves for CFU-S from *src*-infected cultures. Lethally irradiated B6D2F1 mice were reconstituted with nonadherent cells from *src*-infected long-term bone marrow cultures. The input inoculae CFU-S content is given in Table 4. At different times after inoculation, two to three mice were sacrificed and their pooled spleen cells were assayed for CFU-S content. Src 1 to 32 weeks, □——□; Src two to seven weeks, ○——○; Src, 2 to 15 weeks, △——△. Error bars were calculated based on the SEM.

cells.[25] Thus, the sample was taken at the transition between rapid increase in CFU-S and stabilization. This may explain the long CFU-S doubling time. *Src* 2 (15 weeks) was taken during the plateau phase, during which the level of CFU-S was stable and 50- to 100-fold greater than that of the control cultures. The doubling time for these CFU-S was similar to that observed for fresh BM.[21] *Src* 1 (32 weeks) was taken late in the infection during the early part of the decline in CFU-S due to the transformation of the adherent stromal layer. These cultures ceased active hematopoiesis within three to four weeks following this sample.[14,25] The shortening of the doubling time could be a response to the early phase of this hematopoietic decline.

The doubling time calculated from these growth curves is the product of two components, the cell cycle time and the *P* value. Using the assumptions above and cell cycle times similar to those calculated in the previous section, corresponding *P* values may be calculated (Table 5). These *P* values display a very wide variation, as expected from the variation in the doubling times. The variation contrasts sharply with results from normal BM, and suggests that there is a looser control of these parameters in the CFU-S from the *src*-infected cultures.

Table 5. Calculated Data From In Vivo Growth Curves

	Input CFU-S per Mouse	Doubling Time of CFU-S* (h)	*P* Value for Times	
			Tc, 8 hr	Tc, 10 hr
Src 2 at 7 wk.†	1170.0	79.0	0.55	0.56
Src 2 at 15 wk	67.0	24.0	0.67	0.71
Src 1 at 32 wk	37.5	15.7	0.75	0.82

*Doubling time for CFU-S was calculated from the slope of the growth curve in Fig 2.

†Time is given in weeks after infection of the culture with the virus containing *src*

Long-term regulation of CFU-S in vivo. The hematopoietic cell balance was altered following the infection of LTBM cultures with a virus carrying the *src* oncogene.[14] Part of this change may be explained by the altered properties of the CFU-S derived from these cultures. The capacity for serial recloning of multipotential cells from these infected cultures either in vitro[14,15] or in vivo and the altered regulation of *P* values and cell proliferation kinetics suggest that there may be a modification in the response of these CFU-S to normal regulatory factors or environment. Because the altered response results in the overproduction of the CFU-S relative to the differentiated cell types, it might be anticipated that in vivo inoculation of these cells could lead to leukemia. However, neither leukemia nor production of leukemic cells which could be amplified in LTBM cultures has been observed in normal, sublethally or lethally irradiated, syngeneic hosts.[14,25] This failure to induce leukemia suggests the presence of in vivo regulatory factors which are capable of compensating for the defect demonstrated in vitro.

To investigate the long-term effects of the inoculation of CFU-S from *src*-infected cultures in vivo, the growth of CFU-S in the spleens of irradiated recipient mice reconstituted with these CFU-S was followed for a period of 60 days. A smaller inoculum of CFU-S from *src*-infected LTBM cultures in comparison to control cultures was required to reconstitute the lethally irradiated recipients. Figure 3 shows that the growth curves for both inoculae produced nearly identical growth curves, except that the plateau CFU-S level established in the spleen was ~50% higher for CFU-S from the *src*-infected cultures. The ability of cells from the *src*-infected cultures to establish a steady-state level of CFU-S in the recipient animals implies that the growth is regulated in

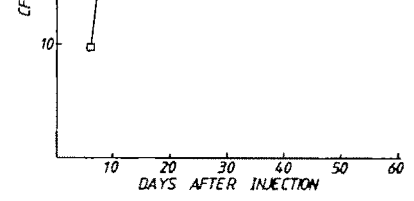

Fig 3. Long-term growth curve for CFU-S from *src*-infected and uninfected cultures. Lethally irradiated B6D2F1 mice were reconstituted with nonadherent cells from either control or *src*-infected long-term bone marrow cultures. Two to three mice were sacrificed for each time point and CFU-S assays were performed on their pooled spleen cells. Values on the *y* axis indicate input levels of CFU-S used to reconstitute the mice. Control CFU-S, ○——○; CFU-S from *src*-infected cultures, □——□.

vivo in the long-term, which may explain the failure of these cells to induce a leukemia.

These growth curves were not corrected for the decreased seeding efficiency of the CFU-S from the *src*-infected cultures (Table 1). However, when the seeding efficiencies of CFU-S taken from the spleens of these reconstituted animals was measured two months after reconstitution, no difference was found between animals reconstituted with CFU-S from control cultures and CFU-S from *src*-infected cultures. This suggests that the altered CFU-S phenotype induced following infection of the LTBM cultures with the *src* virus reverted to a more normal phenotype during the long-term reconstitution.

DISCUSSION

Infection of the LTBM cultures with the murine virus construct containing the *src* oncogene derived from Rous sarcoma virus resulted in an altered balance of the normal cell types present in the cultures.[14,25] There was an increase in the proportion, as well as in the absolute level, of cells which produced normal spleen colonies in irradiated recipients (CFU-S) and in cells which produced colonies in agar using WEHI-3 cell-conditioned medium as a source of colony-stimulating factor. Additional analysis of the founder cells for these agar colonies has revealed that they are not the same as the cells from normal BM which produce colonies using these assay conditions, in that agar colonies derived from the *src*-infected cultures could be serially recloned and were capable of producing CFC-mix colonies.[26] These data suggest that the CFU-S or multipotential stem cells present in the *src*-infected cultures were altered as a consequence of the infection. In previous experiments using the various murine retroviruses, no similar alterations at the stem cell level have been observed,[10] suggesting that in some way the expression of the *src* oncogene is responsible for these alterations. The data presented here provide additional evidence that *src* induces an alteration in stem cell properties.

The possibility that the cell surface properties of the CFU-S from the *src*-infected cultures were altered using the seeding efficiency assay. It was expected that the efficiency with which CFU-S would seed the spleen would be related to properties of the cell surface. This supposition was supported by the reduction of seeding efficiency observed following the removal of sialic acid from the cell surface glycoproteins and/or glycolipids with neuraminidase.[18,19] It was found that the seeding efficiency for the CFU-S from the *src*-infected cultures was about sixfold lower than that for the CFU-S from the control cultures. Treatment with neuramidase reduced the seeding efficiency for control CFU-S by an additional fivefold and for CFU-S from the *src*-infected cultures by threefold, implying that the CFU-S from the *src*-infected cultures may have an altered cell surface. Indeed, alterations in cell surface glycoproteins and glycolipids have been a regular finding in cells transformed by viruses containing *src*.[27-29] The reduced seeding efficiency for the CFC-S from *src*-infected cultures also means that the previously published measurements of CFC-S from these cultures were underestimates.[14] Use of the measured seeding efficiencies to calculate the actual number of CFC-S from the previous CFU-S measurements reveals that the increase in CFC-S in the *src*-infected cultures was 500-fold. At late culture times, this would mean that about 30% of the total nonadherent cells were CFC-S, whereas only 15% were morphologically mature granulocytes. Thus, the normal hierarchy of cell type distribution appears to be reversed, with a predominance of the more primitive types.

Because little is known about specific molecular markers for stem cells or CFC-S, the experiments focused on the functional properties which serve to define stem cells. Stem cells have the dual properties of self-renewal and multipotentiality which allows for the maintenance of the stem cell population and the ability to produce differentiated cell progeny (ie, the functional cells for the several myeloid lineages). It is clear from the serial recloning of the CFU-S, the ability of the CFU-S to reestablish hematopoiesis when transferred onto an irradiated BM stromal layer, and the previously published data in the in vitro recloning in agar,[14,15] that the CFC-S in the *src*-infected cultures retain the ability to differentiate into the various myeloid lineages and produce functional progeny. The property of self-renewal is described by the *P* value, which represents the probability that each daughter cell following cell division will retain the properties and potentialities of the parental cell. For the CFC-S, this means the capacity to found a spleen colony. When the *P* value drops below 0.5, the cell population loses its stem cell character. In the assays of stem cells, the ability to reclone does not depend only on the maintenance of a P value $>$ 0.5—the P value must also be sufficiently >0.5 to overcome any inherent inefficiency in the assay. Furthermore, the CFU-S assay is performed in a potentially lethally irradiated animal, which implies conditions of extreme hematopoietic stress. Previous experiments have demonstrated that CFC-S derived from normal BM or from LTBM cultures are not able to sustain a sufficient *P* value to avoid extinction in serial passage. In contrast, CFC-S derived from the *src*-infected LTBM cultures were able to sustain a high *P* value and to achieve continual logarithmic growth during serial transplantation at short intervals. The unusual ability to maintain high *P* values was also demonstrated more directly by the statistical analysis of CFU-S content of individual spleen colonies. These higher *P* values did not exceed those which have been reported for CFU-S from normal BM. This may suggest that the increased *P* value measured in the irradiated host reflected not an absolute change in the steady-state *P* value but a slower response time to changes in the microenvironment. This type of altered response suggests that the intrinsic change may be caused by an altered sensitivity to regulatory factors. These delayed response times could explain both the altered cell balance obtained in the LTBM cultures and the potential for serial passages and serial recloning.

The short-term CFU-S growth rate in the irradiated host showed remarkable differences, which appeared to depend on the phase of the culture from which the cells were taken, suggesting that the growth rate at these different times is a function of the inoculated cells. A similar conclusion was reached in measuring the growth rate for CFU-S taken from spleen as compared to BM.[24] The variation was interpreted in terms of a mixture of CFU-S with different inherent growth rates. The alterations in rate observed for the CFU-S from the *src*-infected cultures exhibited a greater oscillation than for the normal CFU-S and appeared to change in response to culture conditions. The growth rate was decreased in cells from cultures which had undergone a recent proliferation and which produced a relative increase in CFC-S of about 500-fold, and was highest in the late phase of cultures in which the total CFU-S was starting to decline due to failure of the stromal cell layer.[25] These results suggest that the differences in CFU-S growth rate may reflect the responses of the whole CFU-S population to external factors. The 500-fold increase in CFC-S followed by an exaggerated drop in CFU-S growth rate suggests that a component of the altered regulation could be a delay in the response to overproduction and perhaps also underproduction of CFC-S.

The altered self-renewal and proliferation of CFU-S from the *src*-infected cultures, which could be demonstrated either in the in vitro assays or in irradiated hosts prior to stable reconstitution, appeared to be lost in the later phases of the reconstitution. The longer term growth curves demonstrated little difference in the CFU-S content of spleens of irradiated animals inoculated with cells from control or *src*-infected LTBM cultures by 10 to 14 days after inoculation, and reached similar plateau values. This stabilization is consistent with our previous failure to demonstrate that cells from the *src*-infected cultures were leukemogenic.[14,25] Most of the animals display a mild anemia, as demonstrated by a reduction in their hematocrit. This apparent numerical stabilization of the CFU-S suggests the establishment of an intermediate stage in the development of leukemia and bears some analogies of the situation of human chronic myelogenous leukemia.[30] In this disease, it is apparent that several alterations occur, often sequentially, and give rise to a succession of clinically recognizable states culminating in an acute blast crisis.[30]

Although the demonstration of some intrinsic alteration in the CFU-S in the *src*-infected LTBM cultures would be most easily explained by the infection of these cells by the virus containing the *src* oncogene, this may not be the case. Factor-dependent cell lines derived from *src*-infected cultures remained multipotential, although they do not produce spleen colonies (Spooncer and Dexter, unpublished observations), and contained an integrated Moloney leukemia virus, which was used as a helper virus for the defective *src* virus; however, integrated *src* virus could not be detected (Stoker, Wyke, Spooncer, and Dexter, unpublished observations). Thus, the alterations in the CFU-S described here may be induced by a "hit and run" type of mechanism, which would also help explain the failure of these cells to induce leukemia. In this respect, it is worth noting that although the factor-dependent cell lines derived from the *src*-infected cultures carry the Moloney leukemia virus, this tissue culture-passaged virus is only weakly leukemogenic in adult animals with the C57Bl/6 genetic background.[31] This may help explain the absence of leukemia during the six-month observation period following the inoculation of these cells.

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Inherited Deficiency of Protein S in a Japanese Family With Recurrent Venous Thrombosis: A Study of Three Generations

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We found a new thrombophilic tendency in a family with protein S deficiency. The propositus, a 38-year-old Japanese man, is an offspring of consanguineous marriage and suffered from recurrent episodes of thromboembolism. Hemostatic studies, including platelet counts, platelet aggregation, assays of coagulation factors, and plasminogen activity were all within normal limits. The levels of antithrombin III, α_2-macroglobulin, protein C, and protein C inhibitor were also normal. However, functional protein S activity in plasma was markedly decreased (9%) in the propositus. The family study revealed that the reduced levels of functional protein S, <5% to 29% of normal (normal range: mean ± 2 SD of 15 normal adults were 44 to 180%), were found in 11 members of this family over three generations. Six of 11 members had severe protein S deficiency (<5%), whereas five had partial deficiency. Four of eight adults with protein S deficiency had recurrent episodes of thrombosis. Immunologic levels of protein S antigen were variable in this family and did not correlate closely with the functional levels. These results suggest that the recurrent thrombotic disease in this family appears to be associated with an inherited deficiency of functional protein S.

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PROTEIN S is a vitamin K-dependent plasma protein[1] which may be required for the optimal function of a potent inhibitor of blood clotting and profibrinolytic agent, activated protein C (APC). APC selectively inhibits the coagulant activity of factors V and VIII,[2-4] and also appears to stimulate fibrinolytic activity.[5] Protein S and activated protein C can form a lipid-bound complex that can inactivate factor Va more rapidly than does soluble activated protein C.[6] Thus, the anticoagulant action of APC was not fully expressed in plasma that had been depleted of protein S.[6] When protein S was added back to the depleted plasma, the anticoagulant activity of APC was restored. The hereditary deficiency of protein C is now known to be associated with frequent episodes of spontaneous venous thrombosis.[7,8]

These studies suggest that deficiency of protein S may also be associated with thrombosis. This article describes inherited deficiency of protein S in a Japanese family over three generations, associated with recurrent thrombosis. The findings of our study have been presented in abstract form.[9] After our study was accomplished, similar observations were reported independently from other laboratories.[10-13]

MATERIALS AND METHODS

Plasmas. Venous blood was collected in one-tenth vol of 3.8% sodium citrate. Platelet-poor plasma was obtained by centrifuging citrated blood at 3,000 g for 20 minutes at 4 °C. Normal pooled plasma was composed of 21 normal adult plasmas and was used as a standard plasma in the following assays. Normal pooled plasma was defined as containing 100% activity or antigen. All plasmas were stored in aliquots at −70 °C until use, except that factor V activity, factor VIII activity, and euglobulin lysis time were measured on fresh plasmas within two hours after blood collections.

Routine hemostatic tests. Bleeding time, platelet aggregation, euglobulin lysis time, FDP, antithrombin III activity, and plasminogen activity were performed as previously described.[14] Prothrombin time, activated partial thromboplastin time (APTT), and the procoagulant activities of individual clotting factors were assayed as described earlier.[15]

Protein S, protein C, protein C inhibitor, and their antisera. Protein S, protein C, and protein C inhibitor were purified as reported earlier.[16-18] Rabbit antiserum against protein S,[19] rabbit antiserum against protein C inhibitor,[18] and mouse monoclonal antibody against protein C[20] were prepared and characterized as previously described. The IgG fraction of the antiserum was isolated and $F(ab')_2$ fragments were prepared by pepsin digestion.[21]

Assay of protein S antigen. Plasma protein S was determined by conventional Laurell rocket immunoelectrophoresis. Agarose (1%) (Agarose L, Hoechst, Behring Institute) dissolved in the buffer consisting of 0.05 mol/L of sodium barbital, 0.05 mol/L of Tris, 0.1 mol/L of glycine, containing 5 mmol/L of EDTA, was mixed with antiprotein S serum (0.5% final) at 50 °C. A 1 mm thick gel was poured on polyester filter (GelBond, FMC Corp, Marine Colloids Div, Rockland, Me). Ten-microliter samples were applied and electrophoresed at 1.5 mA/cm at 10 °C for 18 hours using the same buffer containing 5 mmol/L of EDTA. After the electrophoresis, the agarose plate was washed in 0.15 mol/L of NaCl for at least 12 hours and then in distilled water for one hour. The plate was then dried and stained with Coomassie Blue R-250 (0.05% dye in 10% methanol and 10% acetic acid). Under these conditions, the height of the standard purified protein S of 60, 30 and 15 μg/mL in the same buffer showed 39.5, 24.4 and 15.8 mm, respectively, on the plate. As compared with these standards, pooled normal plasma (n = 10) was estimated to contain 27 μg protein S per milliliter. Normal level (mean ± 2 SD) from the data of 21 healthy individuals ranged between 66% and 134%, when the pooled normal plasma level was assigned to be 100%. As described by Dahlbäck,[22] the precipitin arc presumably against the complex formed between C_4b-binding protein (C_4bp) and protein S was faintly observed in the larger arc against free protein S. With this technique, it was hard to quantitate differently the complexed protein S and the free protein S in the plasma.

Assay of Protein C antigen. Plasma protein C was determined by sandwich-type enzyme immunoassay (ELISA) which was developed by using two monoclonal antibodies directing towards the different epitopes on protein C molecule.[20] Micro ELISA plate wells

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(Dynatech Product) were coated with the first monoclonal antibody (10 μg/mL in 0.05 mol/L carbonate buffer, pH 9.6) added at a volume of 300 μL per well. After one hour of incubation, unbound materials were removed by washing three times with the phosphate-buffered saline (PBS), pH 7.2, consisting of 0.01 mol/L of phosphate buffer, 0.1 mol/L of NaCl, 1 mmol/L of $MgCl_2$ containing 0.1% bovine serum albumin (BSA) and 0.05% Tween-20. Then, 200 μL of the second antibody coupled with peroxidase (2 μg/mL) and 20 μL of plasma sample (diluted 20 times), in PBS containing 0.5% BSA and 0.05% Tween-20, were added to the wells, and the mixture was incubated for one hour. The wells were then washed three times to remove unbound materials. Substrate solution freshly prepared—2.5 mg/mL ortho-phenylene diamine dihydrochloride) (Nakarai Kagaku, Japan) and 0.018% H_2O_2 in 0.11 mol/L of Na_2HPO_4 to 0.044 mol/L of sodium citrate buffer, pH 5.4, at a volume of 200 μL—were added to the wells, and the enzyme reaction was allowed to proceed for 30 minutes. The reaction was terminated by adding 100 μL of 1 mol/L sulfuric acid. Absorbance of each well was determined at 490 nm using Microplate reader-600 (Dynatech Product). The details of this ELISA were described in a recent article.[23]

Assay of Protein C inhibitor antigen. Plasma protein C inhibitor was determined by the sandwich-type ELISA by using monospecific rabbit anti-protein C inhibitor $F(a, b')_2$ fragment and the $F(a, b')_2$ fragment coupled with β-D-galactosidase which had been prepared by the method of Yoshitake et al.[24] Analysis was performed at room temperature. Flat-bottomed micro ELISA plate wells were coated with the $F(a, b')_2$ fragment (10 μg/mL) in 0.1 mol/L of phosphate buffer, pH 8.0. After two hours of incubation, unbound materials were removed by washing with the phosphate buffer. The wells were then blocked with 200 μL of 10% normal rabbit serum for two hours. The wells were washed three times with the PBS containing 0.1% BSA. Then 100 μL of sample solution, diluted appropriately with the PBS containing 0.1% BSA, was added to the well and incubated for two hours. After the wells were washed twice with the PBS containing 0.1% BSA, 100 μL of the $F(a, b')_2$ fragment coupled with β-D-galactosidase was added and incubated for two hours. Then the washing was performed three times to remove unbound materials. One hundred microliters of freshly prepared substrate solution, 0.1 mg/mL 4-methyl-umbelliferyl-β-D-galactoside (Sigma, St Louis) in the PBS, was then added to the wells and the enzyme reaction was allowed to proceed for 30 minutes. Fluorescence of each well was determined at 360 nm excitation and 450 nm emission using Fluororeader (Dynatech Product). By this ELISA, 10 to 400 ng protein C inhibitor per milliliter was measured with high specificity and reproducibility.

Assay of protein S activity. Assay of protein S activity was estimated by measuring the ability of protein S to function as a cofactor of the anticoagulant effects of activated protein C. One hundred and twenty-five microliters of protein S-deficient plasma was mixed with 25 μL of test plasma for one minute at 37 °C, and 100 μL of Actin (Dade, Miami, Fla) was added to the mixture. Then, 20 μL of activated protein C (50 μg/mL) was added and incubated for one minute at 37 °C, followed by the addition of 100 μL of 25 mmol/L of $CaCl_2$ to initiate clotting. The standard curve was prepared by substituting test plasma with protein S-depleted plasma containing various amounts of normal pooled plasma (0%, 20%, 40%, 60%, and 80% normal protein S levels). The clotting time was proportional to the amount of protein S in the test plasma. Protein S-depleted plasma was prepared as follows. Thirty milliliters of pooled normal plasma was mixed with 50 mg of anti-protein S IgG-coupled Sepharose 4B (25 mL) of 4 °C overnight, and the plasma was then removed from the agarose by centrifugation at 2,000 rpm for ten minutes. The protein S-depleted plasma was stored at −80 °C until use.

RESULTS

Family report. The propositus (III-3 in Fig 1) is a 38-year-old Japanese man with recurrent thrombosis and pulmonary embolism. He was well until January 1981, when he had painful swelling of his right leg. Ten days later, he also developed similar swelling in his left leg and was diagnosed as having bilateral common femoral vein thrombosis by venograms. Subsequently, similar episodes of a deep vein thrombosis of the right leg occurred frequently (once every two to six months), and he was hospitalized each time and treated with IV urokinase. In November 1983, the patient was involved in a traffic accident and sustained an injury to his left shoulder. He experienced painful swelling of his left arm, and a diagnosis of the left axillar vein thrombosis was made by a venogram. He was treated with urokinase (240,000 U/d) for one week and was then managed with oral anticoagulant (warfarin 3 to 6 mg/d). In May 1984, the patient was admitted to the hospital because of chest pain and was poorly controlled on warfarin (Thrombotest 100% with 3.5 mg warfarin per day). Pulmonary embolism was diagnosed by chest x-ray films and perfusion lung scans. The family history revealed that the patient's parents were first cousins, and that two other siblings had also experienced repeated episodes of thrombotic diseases.

The patient's brother (III-1 in Fig 1), a 42-year-old man, suffered from spontaneous deep vein thrombosis of the left leg confirmed by a venogram at age 24 years. He had similar signs and symptoms in the right leg at age 34 years, and was briefly treated with IV urokinase. The patient's sister (III-2 in Fig 1), a 40-year-old woman, had painful swelling of both legs following childbirth at age 31 years. In December 1976, she was admitted to a local hospital because of inability to walk due to severe pain and swelling of her right leg. She was found to have right femoral vein thrombosis by a venogram.

The patient's paternal aunt (II-4), a 56-year-old woman, had painful swelling of the left calf for one month at age 51 years. The clinical picture of this patient was similar to that described in the earlier two patients (III-1, III-2). She probably had venous thrombosis of the left leg.

The propositus and his family members were studied when they were not taking any oral anticoagulants.

Protein S measurements and other tests. The hemostatic studies of the propositus are shown in Table 1. Platelet count, prothrombin time, APTT, fibrinogen, factor V activi-

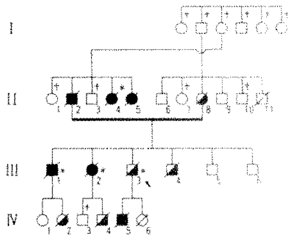

Fig 1. Family with protein S deficiency. The proband is indicated by the arrow. The presence of consanguinity is shown by a solid line between II-2 and II-8. The history of clinical thrombosis is indicated by an asterisk. Severe protein S deficiency (■, ●), individuals with functional levels <5% of normal; partial protein S deficiency (◪, ◐), individuals with 9% to 29% of normal.

Table 1. Hemostatic Studies in the Propositus

Studies	Propositus	Normal or Control
Bleeding time (min)	2	2-5
Platelet count ($\times 10^4/\mu L$)	27.5	16-42
Prothrombin time (s)	12.4	12.7
APTT (s)	29.3	40.1
Fibrinogen (mg/dL)	297	200-400
Prothrombin activity (%)	118	78-140
Factor VII activity (%)	100	75-140
Factor IX activity (%)	108	78-150
Factor X activity (%)	80	70-115
Factor V activity (%)	105	80-130
Factor VIII activity (%)	170	60-170
Euglobin lysis time (hr)	8	1.5-4
FDP ($\mu g/mL$)	10	0-10
AT-III activity (%)	111	80-120
Plasminogen activity (%)	106	85-130
Platelet aggregation (%)		
ADP (4 $\mu mol/L$)	88	55-90
Collagen (3 $\mu g/mL$)	92	65-95
Ristocetin (1.5 mg/mL)	90	55-80
Protein C antigen (%)	70	66-134
Protein C inhibitor (%)	94	59-141
Protein S antigen (%)	38	66-134
Protein S functional activity (%)	9	44-180

ty, factor VIII activity, and platelet aggregation by ADP, collagen, and ristocetin were all within the normal ranges. Anti-thrombin III (AT-III) activity, plasminogen activity, protein C antigen, and protein C inhibitor antigen were also normal. The functional level of protein S, however, was markedly reduced to 9% of normal, since the normal range for 15 normal adults was from 44% to 180% (mean ± 2 SD: 112% ± 68%). Protein S antigen of the plasma of the propositus, measured by Laurell rocket immunoelectrophoresis, was also decreased (38%) (Fig 2). The mean ± 2 SD of protein S antigen in 21 normal adults was 100% ± 34%. The activities of other vitamin K-dependent coagulation factors (prothrombin, factor VII, IX, and X) were all normal. Euglobulin lysis time was moderately prolonged. No evidence of liver disease or disseminated intravascular coagulation was apparent. These results indicate that the patient had an isolated deficiency of protein S.

We then studied the levels of protein S, protein C, and other vitamin K-dependent coagulation factors in plasma of the family members over three generations. Table 2 shows the concentrations of protein S assayed by functional and immunologic methods, and of protein C antigen in 15 members of this family which had been measured on two separate blood collections (July 27 and December 2, 1984). Eight persons including the propositus had markedly decreased levels (37% to 58%) of protein S antigen, whereas all showed normal protein C titers (July 27). In additional plasma samples (collected four months later), virtually identical results were obtained (December 2). In addition to the immunological assay of protein S, we developed a functional assay for the protein S. Eleven of 15 family members had markedly decreased levels of functional protein S, ranging from <5% (the lower limit of sensitivity of this assay) to 29% of normal. Six of 11 persons had severe protein S deficiency, with functional levels <5% of normal. Five individuals including the propositus had partial deficiency of protein S activity (9% to 29%) (Fig 1). Immunological protein S levels of the 11 persons with low functional protein S titers ranged from 36% to 115% and did not correlate with the functional levels. Four of 11 individuals with low functional protein S (II-4, III-1, III-2, and III-3 in Fig 1) had definite clinical histories of thrombosis, whereas the other seven persons, including three children (II-2, II-5, II-8, III-4, IV-2, IV-4 and IV-5), had no such history. The levels of protein C inhibitor, an inhibitor of activated protein C, were within the

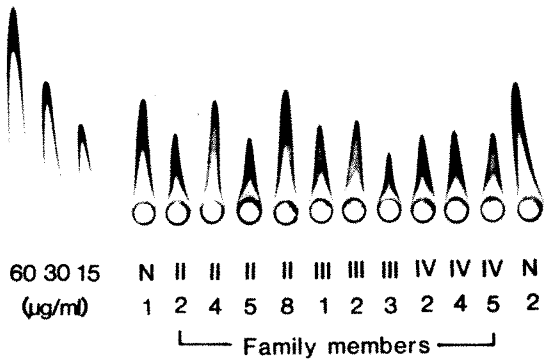

Fig 2. Quantitative immunoelectrophoretic estimation (Laurell's method) of protein S antigen. Wells 1, 2, and 3 contained 60 µg/mL, 30 µg/mL, and 15 µg/mL of purified protein S. Wells N-1 and N-2 contained plasma of normal healthy persons. Wells II-2 through IV-5 contained plasma of affected family members.

Table 2. Concentrations of Protein S and Protein C in Plasmas of Family Members With Recurrent Venous Thrombosis

					Protein S			
			Protein C Antigenic Level (%)		Antigenic Level (%)		Functional Level (%)	Clinical History of Thrombosis
Pedigree Position	Sex	Age	July 27	Dec 2	July 27	Dec 2	Dec 2	
II-2	M	69	92	81	48	49	<5	–
-4	F	56	—	109	—	86	<5	+
-5	F	49	—	70	—	44	<5	–
-8	F	64	146	125	115	107	29	–
-11	M	55	—	70	—	91	70	–
III-1	M	42	113	98	39	44	<5	+
-2	F	40	129	110	58	59	<5	+
-3*	M	38	70	70	38	36	9	+
-4	M	36	152	133	71	69	15	–
-6	M	30	120	109	110	101	ND	–
IV-1	F	17	109	90	88	74	ND	–
-2	F	15	138	112	37	44	11	–
-4	M	8	92	79	45	49	9	–
-5	M	10	—	80	—	44	<5	–
-6	F	8	71	67	67	74	54	–

ND, not determined.

Mean ± 2 SD of protein S antigen, 100% ± 34%; mean ± 2 SD of protein S functional activity, 112% ± 68%; mean ± 2 SD of protein C antigen, 100% ± 35%.

*Propositus.

normal range in plasmas of all members studied (data not shown).

Figure 3 illustrates the activities of prothrombin, factor VII, factor IX, and factor X in plasmas of 15 members of this family. None of these vitamin K-dependent proteins was significantly decreased, and there was no apparent difference between protein S-deficient members (closed circles) and members with normal protein S (open circles).

DISCUSSION

This article describes the hereditary deficiency of protein S in a family with recurrent venous thrombosis. Eleven members over three generations in this consanguineous Japanese family have greatly reduced levels of functional protein S. Four individuals, including the propositus, suffered from thrombosis.

After our study was completed, several Caucasian families with inherited protein S deficiency and thrombotic tendency were described by Comp et al,[10,13] Schwarz et al,[11] and Szatkowski.[12] In one family, two affected brothers had <5% functional protein S activity and 13% to 18% protein S antigen.[10] Six unrelated persons with low protein S activity (15% to 37%) were also found to have recurrent thrombosis.[13] In another report, four members over two generations had 30% to 65% protein S antigen, and all of them suffered from recurrent venous thrombosis and pulmonary embolism.[11] However, the functional activity of protein S was not measured. Two asymptomatic family members had normal protein S levels.

Like Comp et al,[10,13] we found a large discrepancy between functional and immunological protein S titers. Functional levels of affected members were markedly reduced (from <5% to 29%) as compared with immunological levels (36% to 115%). A similar discrepancy was also found in unaffected members (II-2, II-5, II-8, III-4, IV-2, IV-4, and IV-5 in Table 2). Protein S exists in two forms in plasma: as free protein S and as a complex with C_4b; only free protein S appears to be functional in the anticoagulant assay.[13] It was suggested that the Laurell immunoelectrophoresis technique does not distinguish between free and complexed protein S.[13] Although the paternal aunt (II-4) had a normal level (86%) of protein S antigen, she had thrombotic episodes. Her functional protein S titer was <5%. These studies appear to indicate that functional levels of protein S are more directly related to thrombosis than immunological levels of this protein.

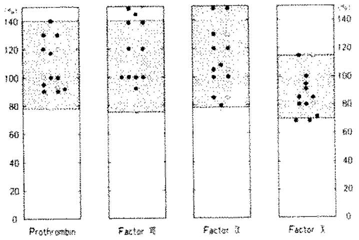

Fig 3. Activities of vitamin K-dependent clotting factors in a family with protein S deficiency. Closed and open circles, individuals with and without protein S deficiency, respectively.

In the present family, 4 of 11 individuals with functional protein S deficiency (<29% of normal) suffered from thromboembolism, whereas two individuals with normal functional protein S did not have thrombosis (Table 2). A chi-square analysis of these data, however, showed that there was no statistically significant relationship between functional protein S deficiency and the occurrence of thrombosis ($P > .05$). Particularly, three members with severe functional protein S deficiency (II-2, II-5, and IV-5 in Fig 1) in this family had no history of thromboembolism. Although one (IV-5) of three members was a child (age <15 years), the other two individuals (II-2 and II-5) were 69 and 49 years old, respectively. Thus, it appears that the severe deficiency of protein S in this family does not always lead to thrombosis. Similar observations were made in familial antithrombin III deficiency, and it is well documented that not all persons with AT-III deficiency develop thromboembolism despite living to an advanced age.[25] What protects these persons is not known at present.

At least three different aspects of our study may be noted. First, we found protein S deficiency in 11 persons over three generations. Functional protein S deficiency is apparently autosomally transmitted (Fig 1). Six of 11 members had severe deficiency (<5% of normal), whereas five persons, including the propositus, had partial deficiency (9% to 29%). The pattern of inheritance in this family may be consistent with an autosomal dominant trait. However, we cannot explain why a son of the propositus (IV-5) has a severe deficiency. More detailed family study is needed to clarify the inheritance pattern of this disorder. Second, it was found that not all individuals with severe functional protein S deficiency had clinically apparent thrombosis. Third, the levels of plasma protein S were found to be consistently reduced over a period of four months in affected members.

The frequency of thromboembolism in Orientals is low. It was reported that the prevalence of thromboembolism in a large group of Japanese examined at autopsy was about one-third of that in a comparable group of Caucasians.[26] Similarly, postoperative thromboembolism is uncommon in Asian populations.[27] Although the reason for the low risk of thrombosis in Orientals is not known, it is interesting to note that hereditary deficiency of protein S in this Japanese family appears to be associated with recurrent thrombosis.

It is not known at present whether the reduced plasma level of protein S in this family is due to either decreased synthesis or increased catabolism. Alternatively, abnormality in C_4bp may lead to increased turnover of protein S. Further studies are needed to answer these important questions.

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α Thalassemia and the Hematology of Homozygous Sickle Cell Disease in Childhood

By M.C.G. Stevens, G.H. Maude, M. Beckford, Y. Grandison, K. Mason, B. Taylor, B.E. Serjeant, D.R. Higgs, H. Teal, D.J. Weatherall, and G.R. Serjeant

α Thalassemia modifies the hematologic expression of homozygous sickle cell (SS) disease, resulting in increased total hemoglobin and HbA_2 and decreased HbF, mean cell volume, reticulocytes, irreversibly sickled cells, and bilirubin levels. The age at which these changes develop in children with SS disease is unknown. Ascertainment of globin gene status in a large representative sample of children with SS disease has afforded an opportunity to study the hematologic indices in nine children homozygous for α thalassemia 2 (two-gene group), 90 children heterozygous for α thalassemia 2 (three-gene group), and 167 children with a normal α globin gene complement (four-gene group). The two-gene group had significantly lower mean cell volumes from birth, higher red cell counts from one month, lower reticulocytes from three months, and higher HbA_2 levels from one year, as compared with the four-gene group. Children with three genes had intermediate indices but resembled more closely the four-gene group. Differences in total hemoglobin or in fetal hemoglobin between the groups were not apparent by eight years of age. The most characteristic differences of the two-gene group were the raised proportional HbA_2 level and low mean cell volume, the latter having some predictive value for α thalassemia status at birth.

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α Thalassemia 2 is common in black populations, heterozygotes and homozygotes occurring in ~30% and 4%, respectively, of Jamaican infants[1] and black Americans.[2] Inheritance of α thalassemia in homozygous sickle cell (SS) disease ameliorates the hematologic features,[3-7] although observations of clinical benefit are less consistent.[4,6]

Among older children and adults, homozygous α thalassemia 2 resulted in higher levels of total hemoglobin, hematocrit, red cell count, and HbA_2, and lower levels of HbF, mean cell volume, reticulocytes, and irreversibly sickled cells. These findings were compatible with reduced hemolysis, which was supported by a prolonged red cell survival.[8] Whether these changes occur in young children is unknown, as is the age at which the changes develop. In the Jamaican sickle cell cohort study, the effects of α thalassemia on the development of hematologic changes in SS disease from birth have been observed.

MATERIALS AND METHODS

Patients. The patients were part of a cohort study of sickle cell disease based on screening of cord blood samples and prospective follow-up of all affected children. During the period from June 1973 to December 1981, 100,000 consecutive normal deliveries were screened at the main Government maternity hospital (Victoria Jubilee Hospital) Kingston, Jamaica, with the detection of 314 children with SS disease, 201 with sickle cell-hemoglobin C (SC) disease, and 47 with the two types of sickle cell-β thalassemia.

Methods. Diagnostic procedures included electrophoresis on cellulose acetate and agar gel.[9] The diagnosis of SS disease was made by the finding of hemoglobin bands in the position of Hb S, F, and A_2. Family studies and the development of HbA_2 levels[10] differentiated children with sickle cell-hereditary persistence of fetal hemoglobin and with sickle cell-β^0 thalassemia. Hematologic procedures[10-12] and methods of follow-up[11] were as previously described.

α Thalassemia status was determined by restriction endonuclease analysis of DNA obtained from the buffy coat of peripheral blood samples.[13] Currently, results are available in 266 children with 9 (3%) homozygous for α thalassemia 2 (α-/α-), 90 (34%) heterozygous for α thalassemia 2 (α-/$\alpha\alpha$) and 167 (63%) with a normal α globin gene complement. Of the 48 SS children without results, 40 were lost before analysis (37 deaths, three emigrated), 6 had defaulted since birth, and 2 children remained to be analyzed. The 266 children with known α globin genotype therefore represented 97% of all surviving SS children in the cohort study. The number of children contributing data to each genotype fell with advancing age (Table 1) because of the age structure of the cohort study.

Hematologic data were entered on a computer, and analysis was undertaken within age groups defined with increasing limits of tolerance as age-related changes became less marked. The birth age group was confined to data collected over 1 to 3 days, 3 months (±1 week), 6 months (±2 weeks), 1 to 3 years (±6 weeks), and 4 to 8 years (±12 weeks) Analysis was limited to steady-state data by excluding hematologic values within 90 days of transfusion; during significant clinical illness (serious infection, acute splenic sequestration, aplastic crisis, painful crisis); or in hypersplenism.

Computer programs calculated mean and SD for each variable at each age group. Data from both sexes were combined in the analysis. The distribution of HbF from one year and of reticulocytes from birth were positively skewed and the logarithmic transformations, $\log_e$ (percentage of HbF + 4) and $\log_e$ (reticulocytes + 1), were used to obtain more normal distributions. Differences between hematologic indices in the genotypes were compared using Student's t test.

RESULTS

Fetal hemoglobin (HbF) fell sharply over the first six months and more gradually thereafter (Fig 1), there being no consistent differences between the different groups. HbA_2 levels increased with age (Fig 2) and were consistently and significantly higher in the three-gene group and highest in the two-gene group, the difference between the two-gene and the four-gene groups being significant at the 0.1% level throughout.

Total hemoglobin levels fell rapidly over the first three months and more slowly thereafter (Fig 3), and no consistent differences occurred between the groups. Mean cell hemo-

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Table 1. Number of Patients Contributing to Data for Each Genotype at Different Ages

	α Globin Genotype		
Age Group	Two-Gene	Three-Gene	Four-Gene
(Total)	(9)	(90)	(167)
6 mo	4	65	113
1 yr	3	55	97
2 yr	6	67	127
3 yr	5	63	120
4 yr	8	65	128
5 yr	7	48	100
6 yr	7	41	90
7 yr	4	31	77
8 yr	5	27	67

globin concentration (MCHC) varied considerably, lowest values tending to occur in the two-gene group and highest values in the four-gene group, but the differences were not consistent or significant. Red cell counts fell sharply over the first month and more slowly thereafter (Fig 4). Between the ages of one month and seven years, values were consistently and significantly ($P < .001$) higher in the two-gene as compared with the four-gene group. Values in the three-gene and four-gene groups were generally similar, although they tended to be lower in the four-gene group. Mean cell volume (MCV) declined with age to six months and then rose gradually (Fig 5). Lowest values occurred in the two-gene group and highest values occurred in the four-gene group, the differences being consistent and significant ($P < .01$) at birth and at all ages. A similar pattern was observed with mean cell hemoglobin.

Reticulocyte counts fell sharply from birth and increased steadily after one month to 36 months. Mean values were lower in the two-gene group as compared with the four-gene group at all ages although the difference was only intermittently significant. Values in the three-gene group were intermediate but similar to those in the four-gene group although a significantly ($P < .05$) lower count did occur at six months. When expressed as absolute reticulocyte counts, values were much more variable and, after logarithmic transformation, levels were significantly lower ($P < 0.05$) in the two-gene as compared with the four-gene group at five years only. No genotype differences occurred in platelet counts or serum iron levels.

In view of the significant differences in MCV between the α globin genotypes at birth, the value of this index in predicting the α globin genotype was examined. The ranges of MCV at birth in the two-, three-, and four-gene groups were 79 to 95: n = 4, 89 to 115: n = 21, and 91 to 120: n = 48 respectively, indicating lower values in the two-gene group but almost identical distribution in the three- and four-gene groups. The 95th percentile (calculated from mean + 1.64 SD) of the two-gene group was 100 fL and the fifth percentile of the 3-gene group was 87 fL indicating a probability of 0.05 that an infant with an MCV of >100 fL would be, and a similar probability than an infant with an MCV of <87 fL would not be, homozygous for α thalassemia 2. Applying this to the 22 children of unknown α globin genotype in whom values for MCV were available at birth, levels exceeded 102 fL in 20 children and were 97 fL in the remaining two, indicating a low probability that any of these individuals were of the two-gene type.

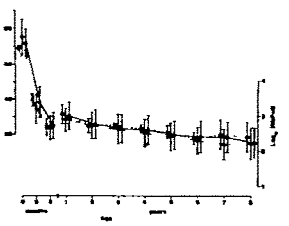

Fig 1. Fetal hemoglobin (mean ± 1 SD) as percentage of HbF from 0 to 6 months and as transformed values ($\log_e$ HbF + 4) from 1 to 8 years. α Globin genotypes: two-gene (■-·■), three-gene (O—O), four-gene (●——●).

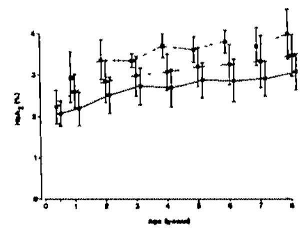

Fig 2. Hemoglobin A_2 percentage (mean ± 1 SD) from six months to eight years. Details as in Fig 1.

DISCUSSION

These observations on the evolution of the hematological changes in SS disease according to α thalassemia status are based on substantial numbers of patients. The 266 children represented 85% of the entire group with SS disease diagnosed at birth and 97% of those surviving. Thirty-seven children died before suitable diagnostic procedures became available, but it is unlikely that this loss seriously biased the prevalence of α thalassemia genotypes since the observed relative proportions agree closely with that predicted from the gene frequency and with figures derived from an older Jamaican population.[4] This loss of children with unknown α globin genotypes renders this data set unsuitable for analysis of survival, although it is adequate for observing the development of hematologic changes within the different subgroups.

Some of the hematologic characteristics of α thalassemia emerge early, significantly lower volumes for MCV and MCH being present at birth.[1] In homozygotes, the red cell

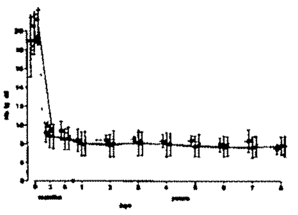

Fig 3. Total hemoglobin in grams per deciliter (mean ± 1 SD) from birth to eight years. Details as in Fig 1.

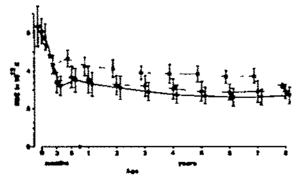

Fig 4. Red cell count times 10^9/L (mean ± 1 SD) from birth to eight years. Details as in Fig 1.

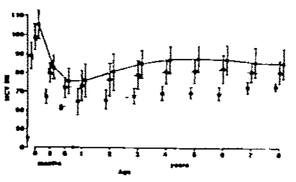

Fig 5. Mean cell volume in femtoliters (mean ± 1 SD) from birth to eight years. Details as in Fig 1.

count was significantly higher at one month, reticulocytes were lower at three months, and HbA_2 was higher at one year of age. It is apparent therefore that most of the hematologic differences characteristic of α thalassemia are apparent from early in life.

Hematologic features in older populations with α thalassemia which have not yet emerged in the cohort study include differences in MCHC, total hemoglobin, and HbF. There was a consistent trend for lower values of MCHC to occur in children with α thalassemia, but because of the variability of values, this trend was not significant. No difference in total hemoglobin levels was apparent by the age of eight years in contrast to the consistently and significantly elevated levels in α thalassemia in previous reports.[3,4,6] This difference is unexplained, although since values for MCHC and MCV were still below levels for their respective genotypes in adults, it is possible that the difference in total hemoglobin will emerge as these indices stabilize in older children. Another possible explanation is that in older patients the difference in total hemoglobin levels was only significant between the two-gene group and those with three or four genes.[4] The difference between levels in three- and four-gene groups in that study was small and not significant, and the lack of expected difference in the present study may be due to the small numbers of patients in the two-gene group.

Data on HbF levels in older populations are conflicting. A small study with five patients homozygous for α thalassemia suggested a significant increase in HbF levels,[3] and the recent report from the American Cooperative Study showed a slight but nonsignificant increase in the 13 patients with homozygous α thalassemia diagnosed by gene mapping.[6] In contrast, a much larger Jamaican study of 44 patients with homozygous α thalassemia, with carefully matched controls, indicated significantly lower values.[4] The magnitude of the reduction was small and was postulated to result from decreased F cell selection secondary to a lower hemolytic rate. Because evidence from reticulocyte counts was consistent with less hemolysis in the present study also, the reason for a lack of difference in HbF by the age of six years is unknown.

It appears that the effects of interacting α thalassemia are generally identifiable within the first few months of life. There is evidence for a reduced hemolytic rate with decreased reticulocyte and increased red cell counts, although no differences in total hemoglobin or HbF are apparent by age eight years. The effect of reduced α globin production is manifest by reduced MCV and MCHC, both of which are theoretically likely to diminish intravascular sickling.[14] The overall pattern of the hematologic features seen in Jamaican SS children with homozygous α thalassemia 2 appears similar to that in children with sickle cell β^0 thalassemia,[15] whereas evidence from older patients suggests that their clinical course is similar.[4]

Clinical data derived from the α globin genotypes in the Jamaican sickle cell cohort study is still at an early stage of collection and analysis. The α globin genotype did not appear to influence the age at which specific symptoms of the disease developed[16] and the effects of alpha thalassemia on splenic complications are complex. There is a suggestion that acute splenic sequestration may be less common in the two-gene group,[17] but preliminary unpublished observations suggest that such patients may be more prone to hypersplenism, consistent with observations in older patients.[4] There is controversy on the role of α thalassemia in survival in SS disease; Mears et al[18] noted a significant excess of three-gene individuals in older patients, whereas Higgs et al[4] were unable to show an increasing prevalence of two-gene individuals with age. These observations could be consistent if the survival benefit of three-gene individuals was compromised by the higher hemoglobin level or other features characteristic of the two-gene group. Unfortunately, because of the unknown α globin genotypes of many of the early deaths, the cohort study cannot contribute to this controversy in relation to early mortality.

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Chromosomal Abnormalities Identify High-Risk and Low-Risk Patients With Acute Lymphoblastic Leukemia

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The importance of banded chromosome analyses in predicting long-term outcome in acute lymphoblastic leukemia (ALL) was evaluated in this follow-up study of 329 patients from the Third International Workshop on Chromosomes in Leukemia. Patients were divided into ten groups according to pretreatment karyotype: no abnormalities, one of the following structural abnormalities [the Philadelphia chromosome, translocations involving 8q24, t(4;11), 14q+, 6q−] or, in the remaining cases, modal number [< 46, 46, 47 to 50, > 50]. Achievement and duration of complete remission (CR) and survival differed among chromosome groups ($P < .0001$). Karyotype was an independent prognostic factor for duration of first CR and survival, even when age, initial leukocyte count (WBC), French-American-British (FAB) type, and immunologic phenotype were considered. Among adults, prolonged remission and survival were uncommon in all chromosome groups. Only in the normal karyotype group was median survival even two years. Among children, striking differences in long-term remission and survival were seen depending upon karyotype. Children in the > 50 group did best, with 70% remaining in first CR for a median duration in excess of five years. Children in the 47–50, 6q−, and normal karyotype groups also had prolonged survivals. In contrast, certain translocations [t(9;22)(q34;q11), t(4;11)(q21;q14–23), t(8;14)(q24;q32)] identified children who had short survivals, even in the presence of favorable prognostic factors including a low WBC, L1 morphology, and non-T, non-B immunologic phenotype. We conclude that chromosome analysis is required at diagnosis in patients with ALL, and that children with these specific translocations should be managed as having high-risk ALL.

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PARTICIPANTS of the Third International Workshop on Chromosomes in Leukemia studied banded chromosomes in 330 newly diagnosed patients with acute lymphoblastic leukemia (ALL).[1,2] Clonal chromosome abnormalities were identified in 218 (66%) patients and, based on the presence of specific abnormalities and modal number, karyotypes were classified into ten groups. Significant differences were found among these chromosome groups with respect to various presenting features, such as age, presenting leukocyte count, distribution of French-American-British (FAB) types and immunologic phenotypes, and with respect to frequency of achievement of complete remission, complete remission duration, and survival. Karyotype pattern was found to be an independent prognostic factor for survival when age, initial leukocyte count, and FAB type were considered.

The Workshop was held in July 1980 and, at that time, 47% of the 330 patients were alive and 33% were still in their first remission. Median follow-up of living patients was only 13 months. We have now studied the influence of longer follow-up on our results. Median follow-up of living patients is now 40 months. Analysis of the data extends our preliminary results to show that karyotype is an independent prognostic factor for duration of first remission as well as for survival. Moreover, we now find that karyotype is a prognostic factor for first remission duration and survival when immunologic phenotype as well as the other risk factors are considered. In addition, we can now with some confidence specify certain chromosome translocations as identifying high-risk patients with ALL, even though other favorable risk factors may be present.

MATERIALS AND METHODS

The series consisted of 330 newly diagnosed patients with ALL studied in 17 institutions. All patients gave informed consent in accord with institutional guidelines. Detailed descriptions of the patient population have been previously reported.[1,2] Banded chromosome studies were performed on material consisting of predominantly leukemic blasts in all cases prior to initiation of treatment. The source of material was bone marrow in 92% of cases; in 63% of cases, direct preparations were studied.

Chromosome abnormalities were designated using the short version of the 1978 International System for Human Cytogenetic Nomenclature.[3] For purposes of this study, an abnormal clone was defined as two or more metaphase cells with identical structural anomalies or identical extra chromosomes or three or more metaphase cells with identical missing chromosomes. For the leukemia to be considered totally normal cytogenetically, at least five metaphase cells from bone marrow had to have been examined and found to be normal.

Patients were divided into ten groups based on chromosome findings. Patients were first classified according to the presence or absence of a clonal chromosome abnormality. Among patients with abnormalities, five recurring structural abnormalities were identified and used for grouping patients: (a) the Philadelphia chromosome (Ph^1), usually as a result of a 9q+;22q− translocation; (b) structural abnormalities involving chromosome 8 at band q24, most commonly an 8q−;14q+ translocation; (c) additions to the long arm of No. 14 (14q+) of other derivations; (d) the 4q−;11p+ or q+ translocation; and (e) a deletion of the long arm of No. 6(6q−). The

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remaining patients with chromosome abnormalities were divided by modal chromosome number into four groups: (a) hypodiploid (< 46 chromosomes), (b) pseudodiploid (46 chromosomes), (c) hyperdiploid A (47–50 chromosomes), and (d) hyperdiploid B (> 50 chromosomes). The cytogenetic and clinical features of these ten groups have been previously summarized.[1,2]

Statistical methods. At the time of initial analysis (September 1980), 155 (47%) patients were still alive. Questionnaires were sent requesting follow-up data on these patients. For each patient, the following data (as appropriate) were obtained: date of first complete remission, date of first relapse, date of death, and date of last follow-up. A minimum of two years follow-up was obtained on all except nine patients. One patient who had originally been considered to have Ph^1+ ALL was reevaluated and found to have chronic myelogenous leukemia and was dropped from analysis.

Status at follow-up was compared among the different cytogenetic groups. Hypotheses of no difference were tested (at $\alpha = 0.05$) by use of the Pearson chi-square statistic for two-way frequency tables (discrete variables). Durations of initial remission and survival of the various chromosome groups were plotted from life tables, calculated by the method of Kaplan and Meier. At the time of this analysis, 22% of the patients were still in remission, and 30% were alive. Thus, differences in remission duration and survival were tested for groups of at least five patients by use of the rank test of Kruskal and Wallis as modified by Breslow for application to censored data. To study the prognostic value of various combinations of risk factors, models of multiple survivorship were examined. The method used was that of Goldman as previously described[1]

RESULTS

Remission duration and survival according to karyotype. Overall, 78% of patients achieved complete remission. The response rates varied significantly among chromosome groups (Table 1, $P < .0001$). With longer follow-up, the differences in duration of initial complete remission among chromosome groups have become more obvious (Table 1). The only chromosome group in which > 50% of patients remain in complete remission is the group with a modal number > 50; the median remission duration for this group will be > 40 months. The shortest median durations of remission occurred in the following groups: t(4;11), four months; 8q24, four months, Ph^1+, eight months; < 46, 8 months.

Among children, striking differences in remission duration were seen ($P < .0001$) (Fig 1). Seventy percent of children in the > 50 group remain in their first complete remission, with a median remission duration that will be > five years. Remissions were very short in the t(4;11) and 8q24 groups (medians of 3 and 4 months, respectively) and moderately short in the following groups: < 46, 13 months; Ph^1+, 15 months; and 46 abnl, 17 months. In contrast, no adults in any chromosome group had long remission durations; only in the > 50 group was the median more than a year (17 months). Statistically significant differences in remission duration among adults were not observed.

Results for survival according to karyotype group were similar to those for duration of first remission (Table 1, Fig 2). Significant differences were seen among the various karyotype groups for all patients ($P < .0001$) and among both children ($P < .0001$) and adults ($P < .0009$) separately.

Influence of karyotype on remission duration and survival when other risk factors are considered. To determine the influence of karyotype on survival when other risk factors were considered, multivariate models of survivorship were compared. Using techniques previously described,[1] we first

Table 1. Remission Duration and Survival According to Karyotype

	Total	N1	>50	47–50	46 Abnl	<46	Ph^1+	t(4,11)	Abnl 8q24	14q+	6q−	*P* Value
All Patients												
Number	329	112	31	28	41	17	38	18	16	15	13	
CR (%)		92	90	77	78	71	54	69	60	53	75	<.0001
Still CR (%)		32	56	35	19	17	15	0	22	13	44	.01
Median CR (mo)*		19	(40)	16	16	8	8	4	4	24	25	.0002
Alive (%)		40	58	32	22	12	8	0	12	20	39	<.0001
Median survival (mo)*		31	(57)	18	22	12	12	7	5	9	29	<.0001
Children												
Number	157	59	22	14	18	7	9	9	6	3	10	
CR (%)		97	95	86	100	100	78	88	83	2/3	80	.17
Still CR (%)		41	70	50	18	29	29	0	0	1/2	50	.09
Median CR (mo)*		27	(60+)	(49)	17	13	14	3	4	—	(26)	<.0001
Alive (%)		53	77	50	33	29	25	0	0	2/3	50	.002
Median survival (mo)*		(50)	(58)	(81)	26	21	15	9	5	—	(37)	<.0001
Adults												
Number	172	53	9	14	23	10	29	9	10	12	3	
CR (%)		87	78	67	61	50	48	50	44	50	1/2	.01
Still CR (%)		22	14	13	21	0	8	0	2/4	0	0	.50
Median CR (mo)*		9	17	10	11	5	7	—†	—	5	—	.47
Alive (%)		26	11	14	13	0	4	0	20	8	0	.22
Median survival (mo)*		24	21	8	10	7	11	7	5	6	—	.0009

CR, complete remission.

*These results are estimated by life-table analysis. Numbers in parentheses are estimated from groups in which ≥50% of patients remain alive

†Medians are not given for <five patients.

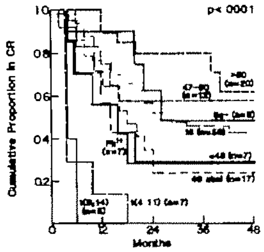

Fig 1. Duration of complete remission of children with ALL according to karyotype of the leukemic cell.

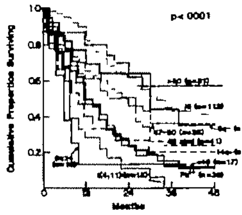

Fig 2. Survival of patients with ALL according to karyotype of the leukemic cell.

evaluated various combinations of risk factors other than karyotype, including age, leukocyte count, percentage of circulating blasts, mediastinal mass, CNS leukemia, FAB types, and lymphocyte surface marker characteristics, to see which combination of risk factors best predicted survival. Age and leukocyte count were grouped into previously identified prognostic classes; each risk factor was treated as a continuous or binary variable.[1,2] Only 154 patients had information on all seven risk factors, including lymphocyte surface markers. The multiple survivorship model containing all seven risk factors was significantly better for estimating survival than was the model which assumed that all patients had the same hazard. However, only age, leukocyte count, and immunologic phenotype contributed significantly to the model (each $P < .05$). For prediction of first remission duration, the best model also included FAB type at diagnosis.

To determine if karyotype added significant prognostic information to age, leukocyte count, and immunologic phenotype for survival, or to age, leukocyte count, immunologic phenotype, and FAB type for first remission duration, karyotypes were grouped as described above and coded as a series of binary variables. Karyotype was first examined for its ability to predict initial remission duration among the 127 patients who achieved complete remission and had complete information on age, leukocyte count, immunologic phenotype, and FAB type. It was then examined for its ability to predict survival in the 173 patients with complete information on age, leukocyte count, and immunologic phenotype. As shown in Table 2, karyotype effectively predicted both remission duration (model 2, $P < .001$) and survival (model 8, $P < .001$). To determine if karyotype was an independent prognostic variable for predicting remission duration, the model (No. 3) containing karyotype and leukocyte count, FAB type, immunologic phenotype, and age was compared with the model containing only leukocyte count, FAB, immunologic phenotype, and age (model 1). Karyotype added significant prognostic information to leukocyte count, FAB, immunologic phenotype, and age for remission duration ($P = .016$). A similar analysis for survival showed that karyotype added significantly to age, leukocyte count, and immunologic phenotype (models 9 and 7, $P = .041$).

A similar analysis was done for the 289 patients who had information on all risk factors except immunologic phenotype. In the absence of data on lymphocyte surface markers, the best model for predicting first remission duration included leukocyte count, FAB type, CNS leukemia, and age (model 4, Table 2). Similarly, for survival, the best model included age, leukocyte count, and FAB type (model 10). Karyotype added significant prognostic information to both of these models (Table 2).

High-risk chromosome groups. Three chromosome groups were associated with short survivals among children as well as adults: t(4;11), 8q24, and Ph^1+. Patients in these chromosome groups were examined in some detail to determine if karyotype or other factors best explained their poor survivals. In each of these three groups, duration of survival was best predicted by specific karyotype.

All 18 patients with t(4;11) have died; only 3 (1 child and 2 adults) survived beyond 16 months, and the longest survivor lived 32 months (Table 3). Survival did not correlate with age, leukocyte count, or FAB type (L1 *v* L2). Treatment appeared to influence response. Of the four patients (nos. 167, 170, 173, and 174) who received induction therapy consisting of only vincristine and prednisone, only one achieved a complete remission, and it lasted for only two months. Eleven patients received regimens consisting of vincristine, prednisone, and either L-asparaginase or an anthracycline or both. Nine (82%) achieved complete remissions which lasted for 2 to 26 months (median 3).

Only four of the 18 patients in this chromosome group had a karyotype other than 46,t(4;11)(q21;q14–23). In two patients (nos. 167 and 172), the material from 4q was translocated to 11p instead of 11q; the only T cell case had a break in 11p15. In two patients (nos. 163, 166), there were additional chromosome abnormalities. Both patients had a mosaic pattern with one clone containing only a t(4;11) with breakpoints in either 11q23 or 25, and a second clone with the translocation and additional aberrations. All three of the long survivors (nos. 163, 166, 172) were among the patients with unusual karyotypes.

Among patients with 8q24 abnormalities, all 6 children and 8 of the 10 adults have died (Table 4). None of the 14 patients who have died survived more than nine months. The presenting clinical and laboratory features of this group were quite homogeneous. Fifteen of 16 patients were FAB L3. Eleven of the 12 patients studied for immunologic phenotype were B cell with surface immunoglobulin present; the phenotype of the leukemic blasts of the remaining patient (no. 178), who was also the one with FAB L1, was pre-B on the basis of intracytoplasmic μ chain immunoglobulin and the common-ALL antigen (CALLA) and lack of receptors for sheep erythrocytes and lack of surface immunoglobulin. Thirteen of the 16 patients were male. The presenting leukocyte counts tended to be low, being $\leq 10 \times 10^9/L$ in

Table 2. Multivariate Analysis of the Prognostic Significance of Karyotype for First Remission Duration and Survival

Model No	Covariates	Models Tested (B-A)	$G^2_A(B)$	df	P Value
		Comparison of Models A and B*			
First remission duration					
1 (n = 127)	WBC, FAB, LSM, age	1-0	41.21	5	<.001
2	Karyotype	2-0	34 06	9	<.001
3	WBC, FAB, LSM, age, karyotype	3-1	20 35	9	.016
4 (n = 217)	WBC, FAB, CNS, age	4-0	71.69	4	<.001
5	Karyotype	5-0	42.51	9	<.001
6	WBC, FAB, CNS, age, karyotype	6-4	20.49	9	.015
Survival					
7 (n = 173)	LSM, WBC, age	7-0	46.9	4	<.001
8	Karyotype	8-0	42.01	9	<.001
9	LSM, WBC, age, karyotype	9-7	17.56	9	.041
10 (n = 289)	Age, WBC, FAB	10-0	92.87	3	<.001
11	Karyotype	11-0	77.05	9	<.001
12	Age, WBC, FAB, karyotype	12-10	33.29	9	<.001

WBC, leukocyte count; LSM, lymphocyte surface markers.

*$G^2_A(B)$ is the chi-square statistic for testing the significance of the improvement in fit of adding the variables which are in model B but not in model A $G^2_A(B) = 2(\ln L(\hat{\lambda}_B) - \ln L(\hat{\lambda}_A))$ where $\ln L(\hat{\lambda}_B)$ is the maximized log likelihood for model B, $\ln L(\hat{\lambda}_A)$ for model A. Model O assumes that the hazard is the same for all patients.

eight patients and 13 to 23 × 10^9/L in eight patients. None of these features correlated with response to treatment or survival (Table 4). The patients were treated with many different standard intensive chemotherapy regimens, including those for Burkitt's lymphoma, other lymphomas, ALL, and acute nonlymphocytic leukemia (ANLL). Nine of the 16 patients achieved a complete remission, but with the exception of the two patients still alive in their first complete remissions at 33+ and 66+ months, these ranged from only 2 to 4 months.

No child survived beyond eight months. Among adults, as in the t(4;11) group, the nature of the chromosome abnormality seemed to be the only factor that distinguished one of the two long-term survivors. Fourteen of the 16 patients had the standard t(8;14)(q24;q32). However, one of the long survivors (patient 188, Table 4) instead had t(X;8)(p22;q24)

Table 3. Clinical and Laboratory Findings in Patients with t(4;11)

Pt No.	Age	Sex	FAB	LSM*	WBC × 10^9/L	Karyotype†	Duration (mos) CR	Duration (mos) Survival
Children								
159	3.5 mo	F	L1	—	668	Standard	0	0
157	9 d	M	L2	—	60	Standard	0	1
158	8 wk	F	L1	N	574	Standard	3	5
160	7.5 mo	M	—	—	214	Standard	2	7
161	14 mo	M	L1	—	58	Standard	3	8
162	16 mo	F	L1	N	151	Standard	8	11
164	7 yr	F	L1	N	248	Standard	3	14
165	11 yr	F	L2	N	449	Standard	2	16
163	18 mo	F	L2	N	4	46,XX,t(4;11)(q21;q25)/ 47,XX,t(4;11)(q21;q25),+mar	16	28
Adults								
168	26 yr	F	—	—	336	Standard	0	0
167	20 yr	M	—	N	20	46,XY,t(4;11)(q21;p12)	0	1
170	28 yr	F	L2	—	392	Standard	0	5
169	27 yr	M	L1	N	436	Standard	0	6
174	47 yr	M	L2	—	20	Standard	0	6
171	32 yr	F	L2	—	250	Standard	4	7
173	47 yr	M	L2	—	28	Standard	2	7
172	40 yr	M	—	T	127	46,XY,t(4;11)(q21;p15)	14	21
166	16 yr	M	L2	N	50	46,XY,t(4;11)(q21;q23)/ 46,XY,−15,+19,t(4;11)(q21;q23)	26	32

*LSM, lymphocyte surface marker type. N, non-T, non-B ALL; —, not done.

†Standard, 46,t(4;11)(q21;q14-23)

Table 4. Clinical and Laboratory Findings in Patients With Breaks at 8q24

Pt. No.	Age (yr)	Sex	WBC × 10^9/L	Karyotype	Duration (mo) CR	Duration (mo) Survival
Children						
179	11	M	9	Standard*	0	1
178†	10	M	10	46,XY,−8,del(3)(q12q25),t(8;14),+der t(1q;8q)(cen;cen)	2	3
175	4	M	10	46,XY,t(8;14)/46,XY,dup(1)(q21),t(8,14)	3	4
177	10	M	16	Standard	2	5
176	6	M	13	46,XY,t(8;14)/46,XY,t(8;14),13q+	4	7
180	12	M	6	Standard	4	8
Adults						
190	65	M	23	46,XY,t(8;22)(q24;q11)/46,XY,t(1;6)(q21;qter),t(8;22)(q24;q11)	0	0
187	41	F	18	Standard	0	1
184	25	M	20	47,XY,+20,t(8;14)/48,XY,+20,t(8;14),+mar	0	2
189	62	M	10	Standard	0	2
183	25	M	17	46,XY,t(8;14)/46,XY,t(8;14),der t(1;14)(q12;q32)	3	4
182	19	M	21	Standard	0	5
181	16	F	6	46,XX,−16,−17,−22,inv(11)(p14q11),t(8;14),+3mar	0	6
185	30	M	4	Standard	4	9
188	54	F	5	46,XX,t(X;8)(p22;q24)	33+	33+
186	36	M	18	Standard	65+	66+

*Standard, 46,t(8;14)(q24 q32). All t(8;14) had the same break points.

†Only patient who was not FAB L3; he had FAB L1 and a pre-B phenotype.

as her only chromosome abnormality. This is the only patient whose translocation with 8q24 did not involve an immunoglobulin gene locus. The explanation for the long survival of patient 186, who had as his only chromosome abnormality a t(8;14)(q24;q32), is unknown; however, surface markers were never done, and the patient was initially diagnosed and treated as a case of ANLL.

In the Ph^1+ group, only three patients (two children and one adult) are known to remain alive. Two of the 38 patients were lost to follow-up at four and 20 months in relapse and have been censored at those times for survival analysis. Overall, patients with the Ph^1+ had a poor survival regardless of age, FAB type, or leukocyte count (Fig 3). Treatment influenced response ($P < .02$). Six patients received only vincristine and prednisone; only one achieved a complete remission. Five patients were treated with ANLL type regimens (daunorubicin, cytarabine ± 6-thioguanine ± prednisone); one achieved a complete remission. However, 26 were treated with vincristine and prednisone plus either an anthracycline or L-asparaginase or both, and 18 (69%) achieved a complete remission. The achievement of a complete remission with any regimen was associated with a longer survival (median 20 *v* 7 months, $P < .0001$). However, all three patients who remain alive in their first remission received vincristine–prednisone–L-asparginase–anthracycline.

Only one of 29 adults in the Ph^1+ group remains alive; she survives in her first remission at 34+ months. However, two of the nine children (1 to 15 years of age) in the Ph^1+ chromosome group remain alive. Both are in their first complete remission and survive 51+ and 63+ months, respectively, from diagnosis. As tended to be the case in the t(4;11) and 8q24 groups, the nature of the chromosome abnormality best identified these two children who were long survivors. These were the only two children who did not have the 9;22 translocation; they were also the only two who had chromosome abnormalities in addiition to the Ph^1. One had five cells studied, all were 47,XY,+1,+10,−19,−21,+del(22)(q11),del(22)(q11); the other had 20 cells studied, four had a normal karyotype, three were 45,XX,−10,del(18)(q21),t(?6;22)(p25;q11), three were 45,XX,−10,?del(1)(q42),del(4)(p12), del(18)(q21),t(?6;22)(p25;q11) and ten cells were 44,XX,−10,−18, t(?6;22)(p25;q11). The importance of the nature of the specific chromosome abnormality, rather than just the modal number, was suggested by the fact that five patients with a t(9;22)(q34;q11) (all adults) had modal numbers of > 50, and none was a long survivor.

DISCUSSION

Our initial analysis found karyotype to be an important risk factor in ALL.[1,2] Karyotype was found to be significantly correlated with achievement of complete remission, duration of remission, and survival. Moreover, karyotype when classified solely according to modal number, the presence or

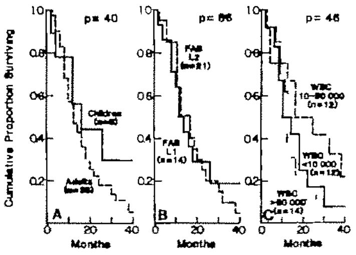

Fig 3. Survival of patients with Ph^1+ ALL according to age, FAB type and leukocyte count at diagnosis. None of these factors significantly influenced the poor survival of these patients.

absence of translocations or more specific chromosome abnormalities as defined above, predicted survival, even when age, initial leukocyte count, and FAB were considered.[1] With longer follow-up, we are now able to show that karyotype independently predicts complete remission duration as well as survival. Moreover, we can now show that for both remission duration and survival, karyotype is a prognostic factor even when immunologic phenotype is considered in addition to age and leukocyte count.

No large study of karyotypes, other than this one, is yet available in adults with ALL. With prolonged follow-up, it is clear that adult patients with any identified abnormal karyotype are unlikely to be long-term disease-free survivors. It is only among adults with a normal karyotype that even 25% of patients still survive and a median survival of two years is seen.

Two other smaller series have also shown that karyotype is an independent prognostic factor in children with ALL.[4,5] Both of these studies have classified karyotype primarily by modal number. The results are generally compatible with ours in that both series find that children with >50 chromosomes often have long survivals, whereas patients with pseudodiploid karyotypes generally have a poor prognosis. However, the modal number classifications used by these other two studies differ from that used here. In our study, only cases that did not have any of five specific chromosome abnormalities were classified according to modal number. Thus, for example, a patient with a modal number of 53 and a t(9;22) would be assigned to the Ph^1+ group, not the hyperdiploid group.

An important aspect of our study is that it suggests that one can more accurately predict response when classification is based on the specific chromosome abnormality. Thus, for example, by classifying cases according to the presence or absence of t(9;22), we identify patients with short survivals even when they have a modal number > 50. This may be particularly important for adult patients, in whom higher modal numbers appear to be seen more commonly in the presence of specific translocations. Moreover, even within our specific chromosome groups, patients who had karyotypes with different breakpoints or rearrangements other than the standard ones often had better prognoses.

In our study, based on the specific translocations, we have identified children who invariably responded poorly to treatment, regardless of other risk factors, and in whom remissions, even when achieved, tended to be short. These included all children with the standard t(4;11), t(8;14), and t(9;22) translocations. These children should be designated as having high-risk ALL, and experimental treatments such as marrow transplantation in first remission should be considered. In contrast, children with other translocations frequently were long-term disease-free survivors (5 of 15 remain in their first complete remission a median of 50+ months). Although several earlier reports (reviewed in reference 6) have suggested that patients with the t(9;22), t(8;14), and t(4;11) have short survivals, our study includes the largest number of cases studied with these translocations and is the only one which directly compares patients with these various chromosome abnormalities, allowing us to designate these cases clearly as consistently having a poor prognosis and one that is worse than that of other patients. Because the number of specific recurring chromosome abnormalities that are associated with distinct clinical-pathologic groups of ALL is rapidly growing (reviewed in reference 7), we believe that the importance of the specific karyotype will increase. Why specific cytogenetic abnormalities correlate with response to treatment and survival requires further investigation.

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Incomplete Antigenic Cross-Reactivity Between Platelets and Megakaryocytes: Relevance to ITP

By Claire P. Stahl, Dorothea Zucker-Franklin, and T.P. McDonald

Immune thrombocytopenias are usually associated with normal or increased numbers of megakaryocytes in the marrow. Therefore, the mechanism(s) responsible for the destruction of circulating platelets may not affect megakaryocytes in the same way. One of the possibilities which could account for the differential effect on the cells would be the development of antibodies to components of platelet membranes which are not exposed on the surface of all megakaryocytes. To investigate this possibility, a rabbit antiserum specific for mouse platelets was tested against fresh and cultured mouse megakaryocytes by indirect immunofluorescence. This antiserum cross-reacted with 46% of fresh murine megakaryocytes and 54% of cultured megakaryocytes. Phase-contrast microscopy revealed the reacting megakaryocytes to be fully granulated with irregular contours and in the process of releasing platelets. Nonreactive megakaryocytes demonstrated smooth contours and lacked morphological evidence of thrombocytopoiesis. Electron microscopy showed that only in megakaryocytes (MK) with an irregular contour had the demarcation membrane system (DMS) reached continuity with the plasma membrane. Ultrastructural analysis of megakaryocytes from patients with ITP showed ~25% to 50% of megakaryocytes without evidence of injury, whereas 50% to 75% had extensive damage. In undamaged cells, platelet territories had not yet reached the peripheral zone. The DMS of damaged megakaryocytes opened to the exterior elaborating platelets. The observations suggested that some platelet antibodies react only with megakaryocytes which have reached the stage of thrombocytopoiesis. Relevant target antigens may not be exposed on all megakaryocytes before cytoplasmic fragmentation occurs.

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THROMBOCYTOPENIA attributed to immune mechanisms is usually associated with normal or even increased numbers of megakaryocytes. This is observed in ITP and in some forms of drug-induced reactions, particularly of the immune complex and passive agglutination types.[1-3] Following the appropriate therapy, the platelet count rises rapidly suggesting that the megakaryocytes are functionally undamaged. In this clinical setting, it appears that antibodies reacting with platelet membranes may not affect a significant portion of megakaryocytes, or may not affect them severely enough to cause complete destruction.

Based on the observation that there is extensive antigenic cross-reactivity between platelets and megakaryocytes,[4-6] it has been generally assumed that platelet and megakaryocyte plasma membranes are virtually identical. However, studies in our laboratory on the process of thrombocytopoiesis using cultured mouse and fresh human megakaryocytes have demonstrated fully developed platelet territories in the cytoplasm of the megakaryocytes with few extensions of the demarcation membrane system (DMS) into the peripheral zone of the cell.[7] Thus, platelet demarcation may occur without apparent connection with the megakaryocyte surface membrane, and a significant proportion of platelets could originate in the interior of the precursor cell. This is contrary to the concept of Shaklai and Tavassoli, which proposes that the demarcation membrane system is derived by invagination of the megakaryocyte plasma membrane.[8] However, freeze-fracture analysis has revealed differences in the partition coefficients of intramembranous particles between platelet and megakaryocyte membranes.[7] These data suggest that the platelet membrane may not be entirely derived from the megakaryocyte plasma membrane. Therefore, the two membranes may be expected to express some antigenic differences.

The purpose of this study was to examine, in light of current concepts of thrombocytopoiesis, the in vitro reactivity of megakaryocytes with an antibody made specific for platelets. In addition, ultrastructural analyses of megakaryocytes from patients with ITP were carried out to assess the frequency, extent, and type of megakaryocyte injury which occurs in vivo and which is assumed to be attributable to platelet antibodies.

MATERIALS AND METHODS

Preparation of fresh cells. Bone marrow cells from 8- to 12-week-old male BDF$_1$ mice (Jackson Laboratories, Bar Harbor, Me), were obtained by flushing freshly dissected femurs into Fischer's Medium (GIBCO, Grand Island, NY) containing 15% to 20% horse serum (Flow Laboratories, McLean, VA) Cells were washed three times. Mouse platelet-rich plasma was prepared from heparinized blood and centrifuged at 125 g for ten minutes. Platelets and bone marrow samples were combined for comparative fluorescent studies.

Human megakaryocytes were obtained for electron-microscopic studies from heparinized bone marrow samples of patients undergoing bone marrow examination for diagnostic purposes. Megakaryocytes from patients with a clinical history and subsequent course characteristic of ITP were compared with megakaryocytes from normal bone marrows. The specimens were washed three times with Hanks' solution containing 10% fetal calf serum (FCS) and centrifuged at 250 g for ten minutes to remove most of the erythrocytes. The "buffy coat" was obtained with a Pasteur pipette and placed in 3% phosphate-buffered glutaraldehyde containing 1% sucrose.

Preparation of cultured cells. Continuous liquid mouse bone marrow cultures were established and maintained as described previously.[7,9,10] Approximately 2 × 10^7 marrow cells from isolated femurs of 8- to 12-week BDF$_1$ male mice were pooled in 25-cm^2 plastic flasks (Falcon, Oxnard, Calif) containing Fischer's Medium

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with 20% horse serum (Flow Laboratories), and 1 μg/L Solucortef (Upjohn, Kalamazoo, Mich). Fresh murine marrow megakaryocytes (MK) represented 0.01% to 2.9% of nucleated cells. Cultures were incubated at 37 °C in 5% CO_2 and maintained up to 20 weeks. Each week, half of the medium and nonadherent cells were removed and replaced with an equal volume of fresh medium with 20% horse serum. Removed cells were assayed for total cell count, number of megakaryocytes, and number of platelets. The percentage of megakaryocytes ranged from 1% to 13.9%. However, when all cells were counted, this variability was found to be due to changes in the numbers of neutrophils and monocytes during the 2 to 20 weeks of culture. The absolute number of MK remained constant with a mean of $3.64 \pm 2.97 \times 10^3$/mL ($n = 11$) at weekly time points representing from three to seven flasks each. Similarly, weekly supernatant platelet counts were constant, $1.70 \pm 0.86 \times 10^6$/mL ($n = 11$). These relatively constant numbers of megakaryocytes and platelets confirmed their continuous production, as did the finding on electron microscopy of numerous free platelets as well as megakaryocytes actively releasing platelets throughout the culture period. Because the absolute number of MK and the pool of platelet-producing MK remained stable throughout the culture period, MK cultures of different ages were often combined for these studies to obtain the largest number of cells. No differences in the fluorescence or ultrastructural characteristics of MK obtained from cultures of different ages were found. Cells were harvested for fluorescence and ultrastructural studies between 2 and 20 weeks after initiation of cultures. Cell culture suspensions containing nonadherent cells were collected by centrifugation of the supernatant at 1,000 g for ten minutes. Both the megakaryocyte and platelet containing pellets were resuspended, combined, and washed three times.

Antiplatelet serum. Rabbit anti-mouse platelet serum (RAMPS) was produced as previously described.[11] Mouse platelets were harvested from ~200 male C3H mice by differential centrifugation, washed three times in 1% ammonium oxalate and twice in saline, resuspended into water (5% suspension), and frozen; the purified platelet homogenate was thawed and injected subcutaneously at multiple sites twice each week for three weeks. Seven days after the last injection, the rabbits were bled. The antiserum thus obtained was heated at 56 °C for 30 minutes and absorbed three times with mouse RBC from equal volumes of defibrinated blood to remove mouse antibodies not specific for mouse platelets. RAMPS has previously been shown to cause in vitro platelet aggregation and, in vivo, to cause a marked thrombocytopenia without anemia or granulocytopenia.[11] Thus, there was no detectable cross-reactivity with any other hematopoietic cells. Preparation of isolated $F(ab')_2$ fragments of the mouse antiplatelet antibody was not felt necessary, since mouse megakaryocytes and platelets are known to be devoid of F_c receptors[12].

Fluorescent antibody staining. Fluorescent antibody staining was done by the indirect technique. Cell suspensions were kept on ice. Each specimen was divided in half and stained in parallel with RAMPS and control preimmune serum. Cell suspensions were incubated sequentially with 0.02 to 0.04 mL RAMPS or 0.02 to 0.04 normal rabbit serum for 30 minutes and 0.04 mL of fluorescein-conjugated goat antirabbit globulin (Meloy, Springfield, Va) for 30 minutes. Between steps, the cells were washed three times with phosphate-buffered saline (PBS). After the final wash, the cells were fixed in 2% paraformaldehyde with 0.1% glutaraldehyde for six minutes to preserve cell integrity and to prevent nonspecific interiorization of the label by living cells. On some occasions, the cells were fixed first and were then stained with antibody. The same results were obtained if living cells were fixed following staining. All cells, including megakaryocytes and platelets, were identified initially by phase microscopy and then by fluorescence microscopy. A Leitz Orthoplan microscope with ultraviolet UV optics using epifluorescence and 63x or 100x oil-immersion objectives was used.

Electron microscopy. The specimens intended for ultrastructural analysis were placed in glutaraldehyde and postfixed with osmium tetroxide for two hours followed by en bloc staining with 0.5% uranyl acetate in saline for one hour. Dehydration and embedding in Poly/Bed 812 was carried out as described elsewhere.[7] An LKB ultrotome was used for sectioning, and a Siemens Elmiskop I electron microscope was the instrument used for all studies.

RESULTS

Fluorescence and electron-microscopic analyses. Cell suspensions of fresh as well as cultured murine bone marrow incubated with RAMPS followed by fluorescein-conjugated anti-rabbit Ig showed fluorescent and nonfluorescent MK in almost equal numbers. Megakaryocytes were identified primarily on the basis of their size and irregularly-shaped nuclei. In fresh bone marrow specimens obtained from eight different mice, 53.8% megakaryocytes identified by phase-contrast microscopy did not show any fluorescent staining with RAMPS. Likewise, cells from three preparations of cultured MK showed 46.2% nonreactive megakaryocytes. Platelet clumps were always strongly positive in fresh and cultured marrow specimens, whereas no other hematopoietic cells reacted with the antiserum. In fresh bone marrow suspensions, which always contained large numbers of myeloid and erythroid cells, no fluorescence was observed in cells other than platelets or megakaryocytes. Neither was nonspecific fluorescence seen in suspensions of marrow cultures which have been shown to contain large numbers of monocytes and myeloid cells. No fluorescent cells were seen in identical specimens stained with control serum.

The pattern of fluorescent MK staining was also noteworthy. Although it is well recognized that the fluorescent antibody technique when used on unfixed living cells or on cells fixed in suspension with aldehydes will only detect antigenic determinants on their surface,[13-15] the majority of reacting MK had a diffusely stained or mottled cytoplasm (Fig 1a). Phase-contrast microscopy of fluorescent and nonfluorescent cells and electron microscopy carried out on similar specimens were used to elucidate this phenomenon and to characterize the type of MK which reacted with the antiserum. On phase-contrast microscopy (Fig 1b), the positive cells were seen to have an irregular contour, as is typical for MK which have reached the stage of thrombocytopoiesis.[7] The ultrastructure of a cell comparable to this state of maturation is shown in Fig 1c to illustrate this point. Some MK were entirely nonreactive, whereas others showed fluorescence only on a portion of the rim and adjacent cytoplasm (Fig 2a). Phase-contrast microscopy of the latter cells indicated that the fluorescent areas corresponded to regions where platelets were being released (Fig 2b). The nonstaining areas of the surface membrane were smooth, which is presumed to be a reflection of the lack of continuity of the demarcation membrane system with the surface, as exemplified ultrastructurally in Fig 2c. Occasionally, a positively staining MK with an irregular contour and a negative MK with a smooth periphery were seen side by side (Fig 3). In such situations, the "smooth" MK probably had an uninterrupted periphral zone like the cells whose ultrastructure is illustrated in Figs 2 and 4. The observations indicate that the platelet antibody did not cross-react with all megakaryo-

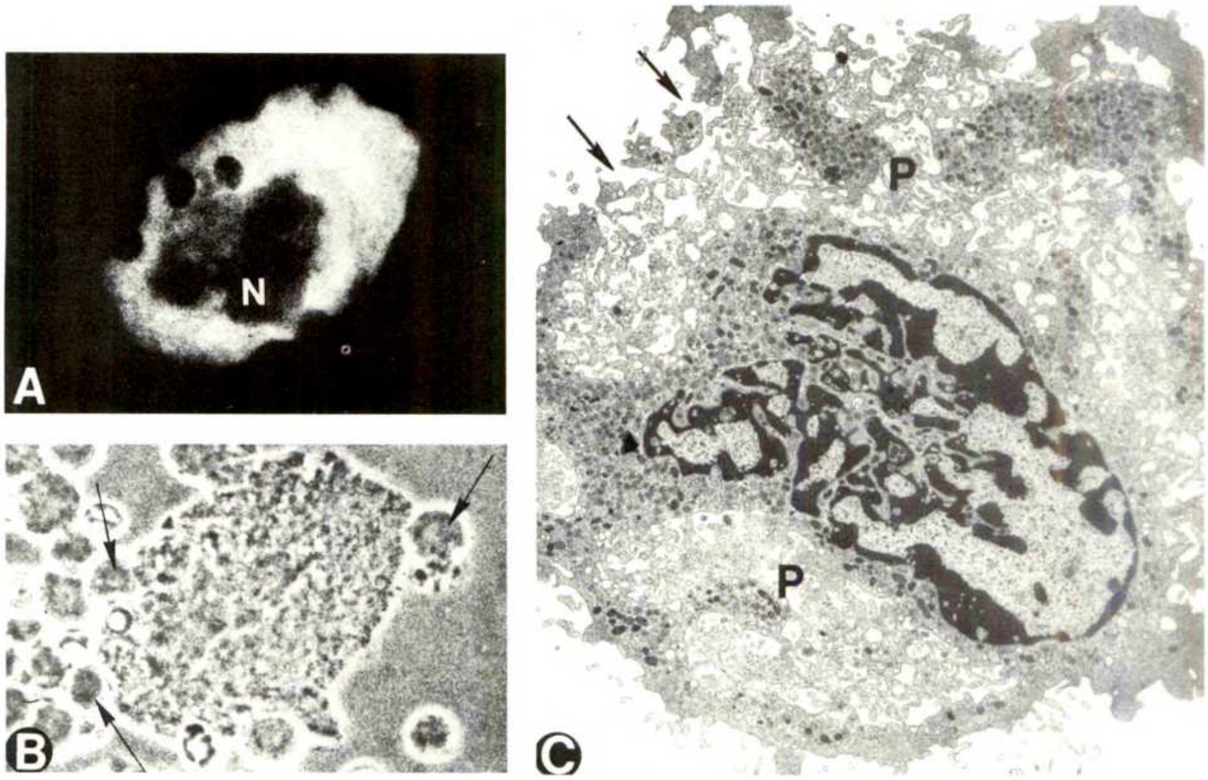

Fig 1. (A) Mouse megakaryocyte (MK) treated in suspension before fixation with RAMPS followed by fluorescein-conjugated goat anti-rabbit globulin. The cytoplasm stained diffusely without clear delineation of a surface membrane. N, nucleus. Apparent indentations are caused by surrounding and overlying nonreactive marrow cells (see B). (B) Phase-contrast photomicrograph of the same cell as depicted in A shows fully granulated cytoplasm and irregular contour characteristic of cells releasing platelets. Arrows indicate other hemopoietic cells which partially cover the MK. (C) Ultrastructure of a mouse megakaryocyte considered to represent the same stage of maturation as the cells shown in A and B. Platelet territories are fully developed (P). The contour is irregular. Antiserum would have access to platelet fields located in the interior without prior fixation of the cell (arrows). Magnification ×3,700.

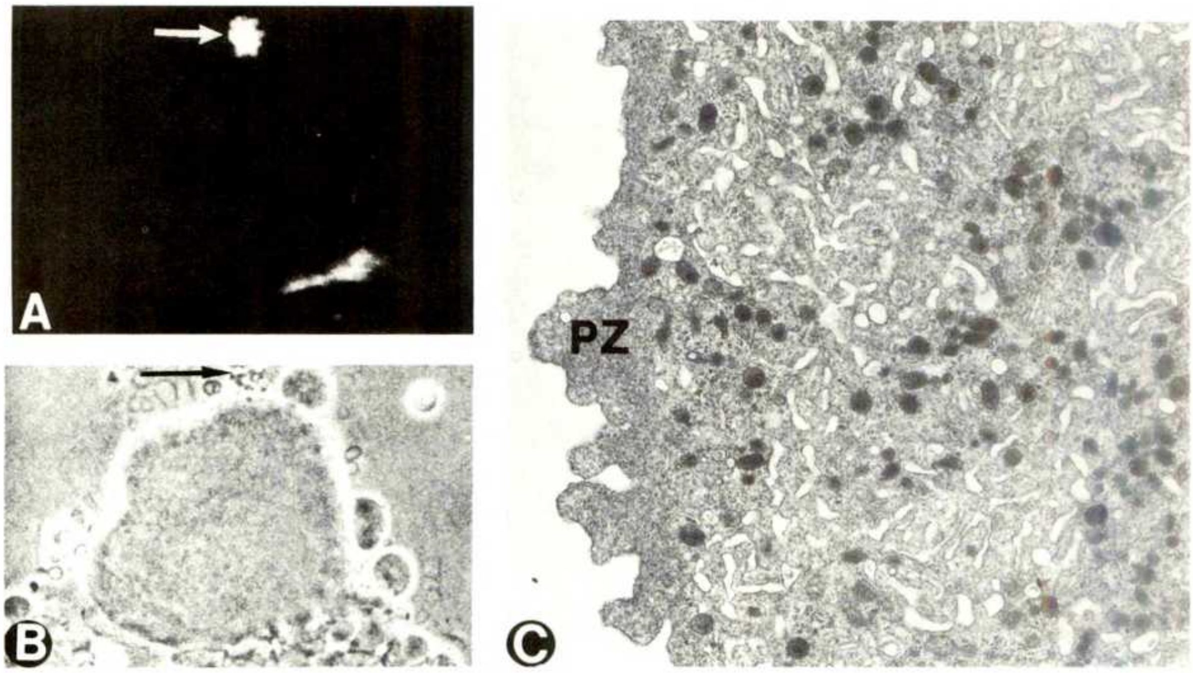

Fig 2. (A) Mouse MK from the same specimen as the one described in the legend to Fig 1A does not fluoresce. With the exception of the small area near the rim (bottom), this cell did not stain with the antiserum. In contrast, the platelet clump at the top is brightly fluorescent (arrow). (B) Phase-contrast photomicrograph of the nonfluorescent cell depicted in A. The contour is smooth except for a small portion at the bottom which corresponds to the fluorescent area in A. Arrow indicates platelet aggregate seen to fluoresce in A. Also shown is the specificity of the antiserum for platelets. The surrounding bone marrow cells are negative. (C) Detail of a mouse MK showing an intact peripheral zone (PZ) illustrating that platelet territories need not reach the surface of the cell. Magnification ×12,000.

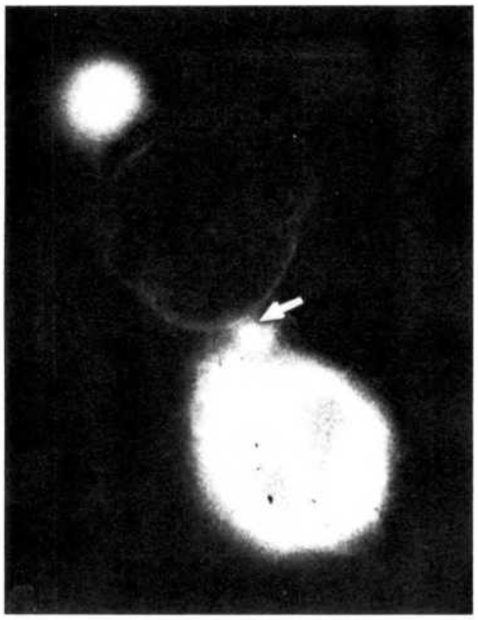

Fig 3. Mouse megakaryocytes treated as described in the legend to Fig 1A. A strongly fluorescent clump of platelets is seen at the top. An MK with diffusely stained cytoplasm and with an irregular contour is seen at the bottom. The arrow indicates a second platelet clump which may represent platelets in the process of being released. Between the fluorescent platelet clump and the fluorescent MK, the outline of a negative MK can be discerned.

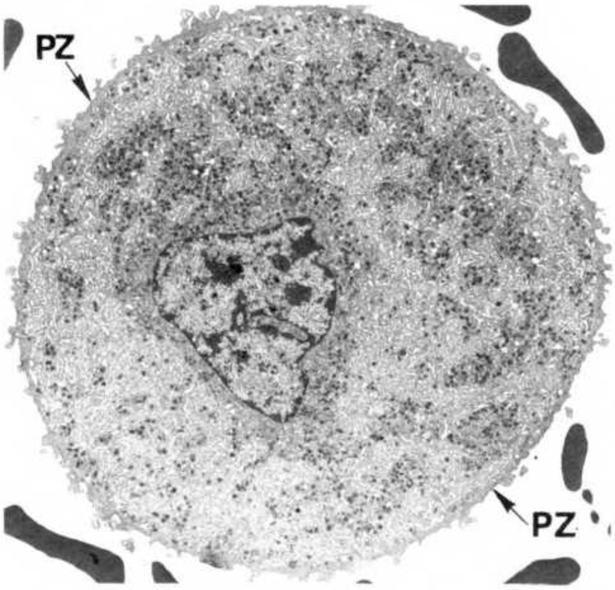

Fig 4. Human megakaryocyte obtained from a patient with ITP. The cell shows no evidence of damage. Platelet territories are fully developed within the cytoplasm. The peripheral zone (PZ) is intact. Magnification ×2,700.

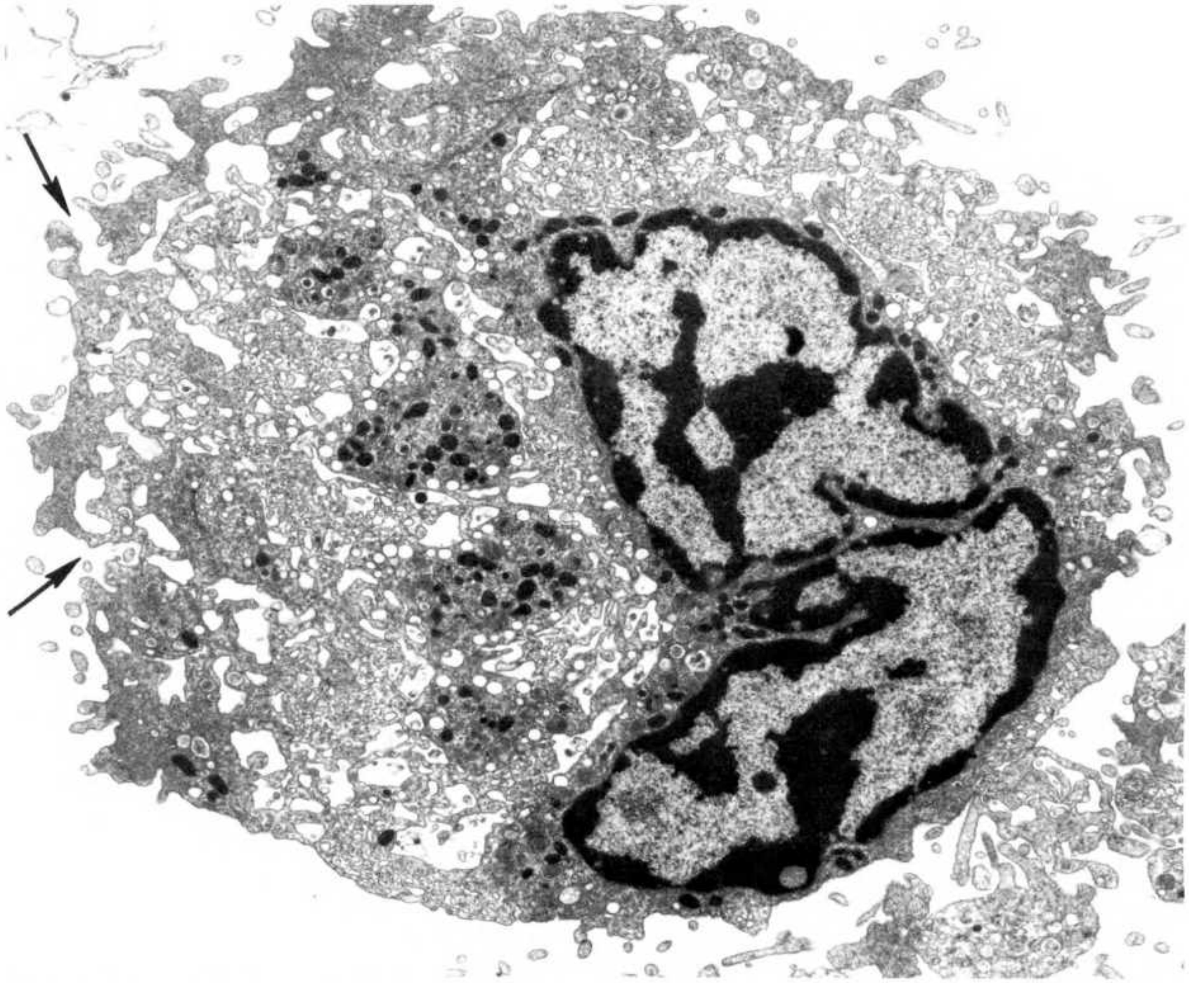

Fig 5. Mouse MK in the process of thrombocytopoiesis. Arrows indicate sites where antiserum could gain access to fully developed platelet territories located in the interior of the cell. The platelets at the bottom are likely to have been released by this MK. Magnification ×17,500.

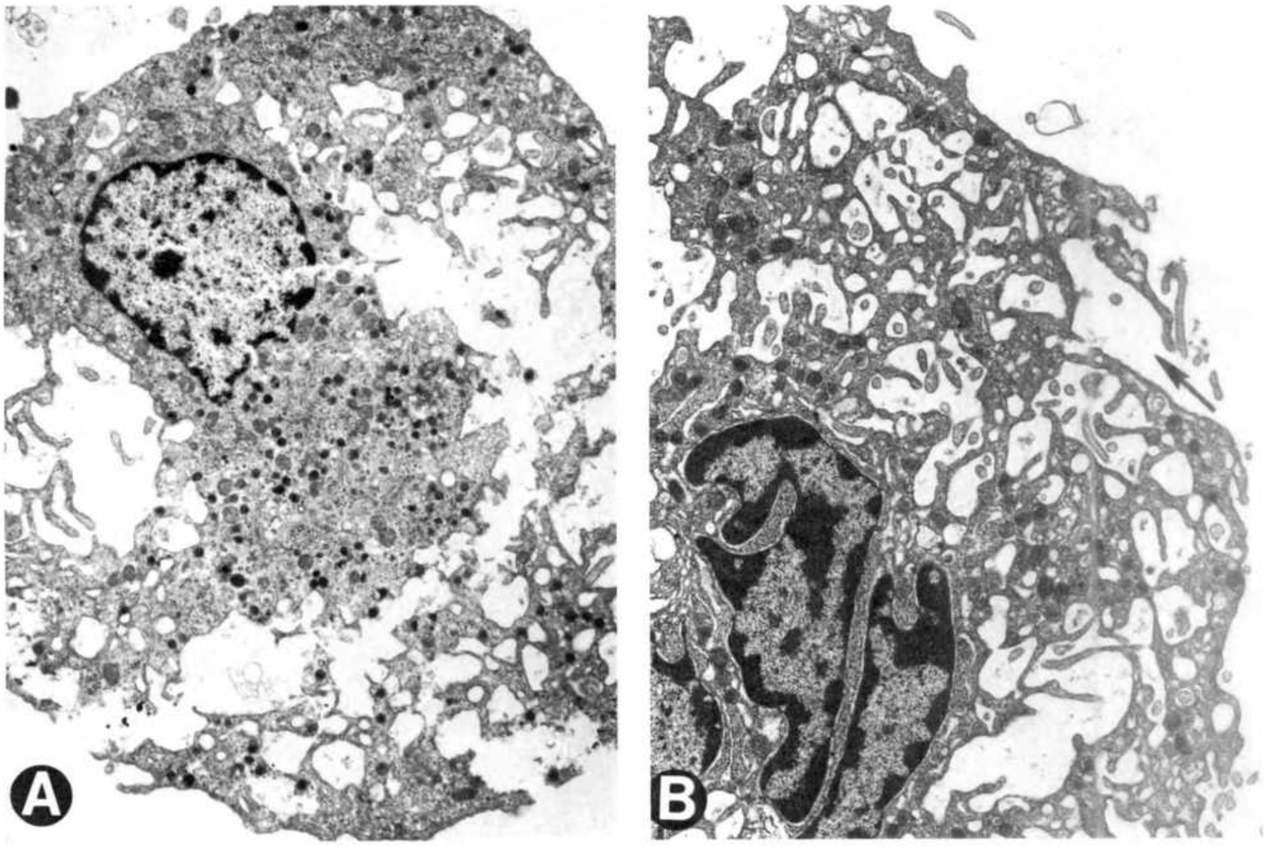

Fig 6. Ultrastructure of megakaryocytes in the bone marrow of a patient with chronic ITP. (A) This cell shows extensive damage and an interrupted peripheral zone. Magnification ×6,000. (B) Detail of MK from the same specimen shown in A. Marked dilatation of the demarcation membrane system is evident. Continuity of extracellular space and demarcation channels are apparent (arrow). Magnification ×7,300.

cytes, but primarily with those releasing platelets, ie, when the membranes delineating platelet territories had established continuity with the surface. In Fig 5, this is illustrated more clearly. When the MK cytoplasm was in the process of fragmenting into platelets, the extracellular medium had access to the interior of the cell. Therefore, in this situation, antisera could reach internal structures without the need for agents that enhance plasma membrane permeability.

Ultrastructure of ITP megakaryocytes. In light of the observations described above and the recognition that patients with severe immune thrombocytopenia may have normal or even increased numbers of MK, we studied the MK of four patients with ITP. Contrary to reports by others,[16,17] we found that about 25% to 50% of the MK of patients with ITP showed no morphological evidence of injury (Fig 4). Normal undamaged cells were observed which had an intact, smooth peripheral zone, devoid of organelles and demarcation membranes, but with a cytoplasm often exhibiting fully developed platelet territories (Fig 4). Such cells were ultrastructurally indistinguishable from the smooth contoured mouse MK (Fig 2c). Approximately 50% to 75% of the ITP MK in well-preserved specimens showed extensive damage. This consisted primarily of a markedly distended demarcation membrane system that made the cells appear vacuolated on light microscopy, as reported by others.[16,17] The surface membrane of injured MK could usually be seen in continuity with the DMS, whereas pieces of MK cytoplasm and/or abnormal platelets had partially or completely detached from the main body of the cell (Fig 6A and B). Pathologic MK were often emperipolesed by mononuclear leukocytes and neutrophils (not shown). Frequently, damaged cells showed attached monocytes, which appeared to phagocytose fragments of megakaryocytes (Fig 7). In specimens examined by us to date, no morphological alterations have been seen in ITP megakaryocytes that had an intact peripheral zone.

DISCUSSION

The studies reported in this communication have shown that a rabbit polyvalent antiserum made specific for mouse platelets cross-reacted with only approximately one-half of the mouse megakaryocytes examined. Subsequent examination of the fluorescing megakaryocytes with phase optics revealed them to be fully granulated, to have irregular contours, and to be in the process of releasing platelets. In contrast, nonreactive megakaryocytes had smooth contours and did not seem to be undergoing thrombocytopoiesis. Ordinarily, immunohistochemical staining of intracellular structures requires permeabilization of the surface mem-

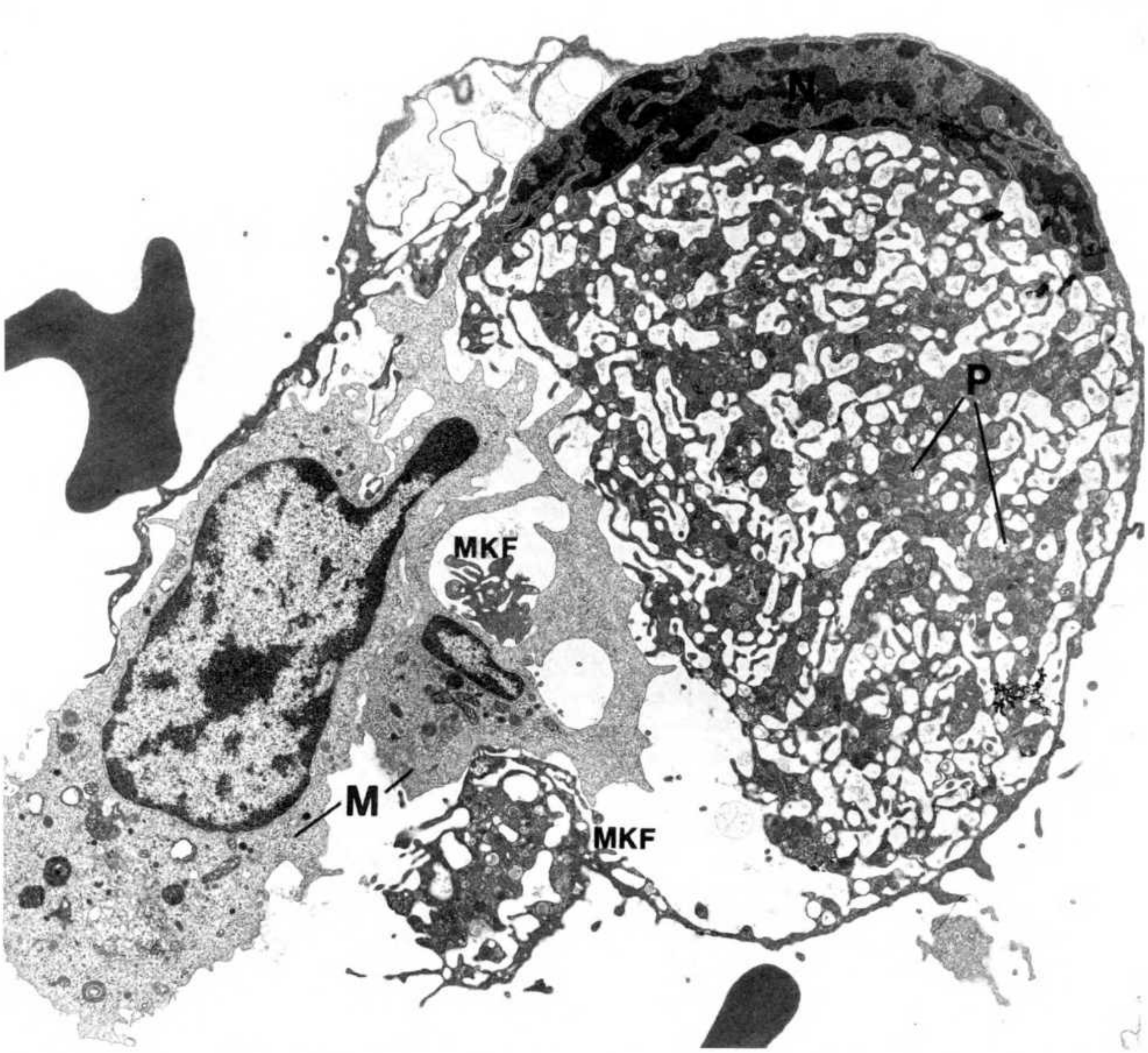

Fig 7. Example of interaction of a "fragmenting" ITP megakaryocyte with other marrow cells. Platelet territories are clearly delineated within the cell's cytoplasm (P). The DMS is markedly distended and in continuity with the extracellular medium. Two monocytes are seen in the process of phagocytosis and/or emperipolesis. The smaller cell appears to have engulfed an MK fragment (MKF). N, nucleus of MK; M, monocytes. Magnification ×6,700.

brane by drying or lipid solvents[18]. It is well recognized that antibodies do not enter living or glutaraldehyde-fixed cells unless they have large openings or invaginations in continuity with the extracellular space, which is the case in fragmenting megakaryocytes. In all likelihood, the smooth, negatively staining cells correspond to those which have been shown ultrastructurally to have an intact peripheral zone (Fig 2c). Conversely, cells with an irregular contour and reactivity with RAMPS represented MK with a DMS extending through the peripheral zone. Electron-microscopic analysis of ITP megakaryocytes seemed to support our hypothesis because only cells that had reached the stage of thrombocytopoiesis appeared damaged. Damaged ITP megakaryocytes had irregular contours and many extensions of the DMS to the periphery (Figs 6 and 7) in continuity with the extracellular medium. ITP megakaryocytes with an intact peripheral zone, in which platelet fields had not yet involved the surface, showed no obvious damage (Fig 4).

Previous investigators, using heterologous anti-platelet antisera and human anti-platelet antibodies conjugated with fluorescein demonstrated antigenic cross-reactivity between platelets and megakaryocytes.[4-6] Our observations are not at variance with these reports. However, by identifying megakaryocytes on phase microscopy before exposing them to UV light, we noted megakaryocytes that were not reacting with the platelet antibody. In the earlier studies cited above, the emphasis was placed on the identification of positively stained cells. More recently, antisera directed against specific platelet proteins[12,19-21] as well as monoclonal antiplatelet antibodies[22,23] have been prepared and tested against MK. Thus, it has been shown that antisera to platelet membrane glycoproteins Ib, IIb, and IIIa, platelet factor VIII:Ag, and fibronectin are reactive with >90% of megakaryocytes analyzed.[12,19] Others, using a monoclonal antibody recognizing an antigen on the platelet membrane glycoprotein IIb/IIIa complex have confirmed these data.[22,23,24] However, when a monoclonal antibody specific for platelet glycoprotein Ib was used, there was much weaker binding of the antibody to MK, and some recognizable megakaryocytes failed to react altogether.[22] These studies leave no doubt that MK and platelets share important antigens, but they also suggest that the antigenic cross-reactivity is not complete. This statement may be particularly relevant to the immune process operative in chronic ITP. Evaluating autoantibody specificity in patients with this condition, Woods et al have found antibodies directed against the platelet glycoprotein IIb/IIIa complex in only 5 of 56 patients,[25] and against glycoprotein Ib in only 3 of 106 patients.[26] Thus, the autoantibodies in this disorder are frequently directed against platelet antigens other than these well-characterized glycoproteins. To what extent any of these autoantibodies react with megakaryocytes is currently unknown. Previous studies evaluating megakaryocyte reactivity with ITP sera have been reported.[27,28] McMillan et al[28] showed increased megakaryocyte binding of purified radiolabeled IgG from serum and cultured splenic cells in two patients with ITP. Using sera preabsorbed with platelets, McKenna and Pisciotta demonstrated cross-reactivity in some patients with ITP but not in others.[27] Various ultrastructural abnormalities in ITP megakaryocytes have been reported before.[16,17] These consisted of vacuoles and a distended demarcation membrane system and the intussusception of other marrow cells.[16,17,29,30] In essence, such studies only confirmed light-microscopic observations[31,32] and phase-contrast data published earlier.[33] Unlike reports which claimed that some patients with ITP had only normal megakaryocytes whereas others had damaged cells,[16] the present study demonstrated heavily injured megakaryocytes alongside entirely normal megakaryocytes in the same specimens. The normal-appearing megakaryocytes had not reached the stage of thrombocytopoiesis, whereas significantly damaged cells were releasing platelets. That the damage had indeed occurred in vivo is further supported by the finding of bone marrow reticuloendothelial sequestration of heavily damaged megakaryocytes. This is not seen in normal bone marrow aspirates obtained for diagnostic purposes.

Taken together, the observation recorded here should be of interest from two points of view. One has to do with the normal physiology of thrombocytopoiesis, the other with the mechanism of immune thrombocytopenic disease. The steps which should be taken to build on the data related to the first subject seem clear. Immunohistochemical studies on the ultrastructural level will be necessary to determine whether some antibodies specific for platelets react only with DMS and/or other internal membranes without affecting the surface membrane of megakaryocytes and at what stage of differentiation such antigens arise. The second area is more complicated. Although it does not seem farfetched to postulate that such antibodies may develop to normal or aberrant platelet membrane components which are not exposed on the surface of megakaryocytes or at least not at every stage of differentiation, the possible heterogeneity of such naturally evolving antibodies may be very great. Such heterogeneity must, however, be taken into account when new modalities for the management of immune thrombocytopenias are contemplated.

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Selective Cytotoxicity of ^{125}I-Labeled Monoclonal Antibody T101 in Human Malignant T Cell Lines

By Epie Boven, Tore Lindmo, James B. Mitchell, and Paul A. Bunn, Jr

The radiolabeled anti-T cell antibody T101 can be used for specific tumor localization, but unlabeled T101 produces limited cytotoxicity in patients. We thus studied the in vitro cytotoxic effects of T101 labeled with ^{125}I, a radionuclide known for its short-range, high-linear-energy electrons. We showed that ^{125}I-T101 could be readily prepared at high specific activity with high immunoreactivity. Human malignant T cell lines HUT 102, MOLT-4, and HUT 78 were found to differ in the number of T65 determinants (the antigen recognized by T101) and the sensitivity to external x-ray radiation, which were of significance for the cytotoxicity of ^{125}I-T101 in vitro. The cytotoxic effects of ^{125}I-T101 were also found to be dose dependent and increased with exposure time under frozen conditions. As controls, unlabeled T101 had no cytotoxic effect, while free Na^{125}I or the ^{125}I-labeled irrelevant antibody 9.2.27 exerted minor cytotoxicity. In HUT 102 and MOLT-4, more than 3 logs' cell killing was achieved within four weeks. Because considerable cytotoxicity was demonstrated in vitro by ^{125}I-T101 on T65-positive malignant cells, and because low-dose ^{111}In-T101 can be used successfully for tumor localization, future trials using ^{125}I-T101 at high specific radioactivity may improve therapeutic results in patients with T65-positive malignancies.

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MONOCLONAL ANTIBODIES have considerable significance as imaging and treatment modalities in malignancies. Several antibodies are already under study in patients with leukemia and lymphoma.[1] T65 is a 65,000-dalton antigen present on the surface of normal and malignant T cells and on nonsecretory chronic lymphocytic leukemia (CLL) cells.[2,3] Monoclonal antibodies recognizing T65 (eg, T101 and Leu-1) have been administered to patients with CLL and cutaneous T cell lymphoma (CTCL).[3-8] Although T101 bound to malignant cells in the circulation, bone marrow, lymph nodes, and skin, the treatment usually resulted in limited clinical benefit manifested by a transient drop in circulating malignant cells. It was also demonstrated that modulation of the T65 antigen on target cells occurred in vivo and in vitro, limiting the effectiveness of prolonged infusions of unlabeled antibody.[9,10] Because the antibody could be administered safely and reached tumor cells in tissue sites, the conjugation of radionuclides, cytostatic agents, or toxins has been considered for therapeutic purposes. In this respect, internalization of the antigen–antibody complex may be of high value.

Radiolabeling of T101 with ^{111}In has been successfully achieved, and radionuclide scanning of patients with CTCL demonstrated specific uptake in tumor lesions located in lymph nodes and skin.[11] Some of these lesions had not been detected by conventional noninvasive procedures. Clinical studies in radiotherapy are in progress, including the application of ^{131}I-labeled antibodies at high specific radioactivity in hepatoma and melanoma.[12-15]

By use of ^{125}I, a radionuclide with unique subcellular radiation properties, conjugated to monoclonal antibody T101 at high specific radioactivity, we demonstrate that substantial radiation damage can be reached in vitro in cell lines expressing the T65 antigen. This radiation damage is specific and dose dependent, increases with exposure time, and is further dependent on antigen density and radiation sensitivity of the target cell. Further therapeutic studies with ^{125}I-T101 seem to be justified in patients with T65-positive malignancies.

MATERIALS AND METHODS

Cell lines and monoclonal antibodies. Malignant T cell lines HUT 102 and HUT 78 were initiated in our laboratory and previously described.[16,17] HUT 102 is a human T cell leukemia virus (HTLV)-positive line derived from an adult T cell leukemia/lymphoma patient. HUT 78 was derived from a CTCL patient and is HTLV negative. MOLT-4, a T lymphoblastoid leukemia line, was obtained from American Type Tissue Collection, Rockville, Md. The cell lines, growing in floating aggregates, were maintained in RPMI 1640 medium (GIBCO, Grand Island, NY) and 10% heat-inactivated fetal bovine serum (FBS) (GIBCO). Melanoma cell line NCI-N892 was initiated by D.N. Carney in our laboratory and was growing in monolayer culture in the same medium.

Monoclonal antibodies T101 and 9.2.27 (Hybritech Inc, La Jolla, Calif) are murine IgG$_{2a}$. T101 recognizes the T65 antigen, which is present on HUT 102, MOLT-4, and HUT 78. Monoclonal antibody 9.2.27 has a high specificity for human melanoma cell surfaces and recognizes a 250,000-dalton antigen,[18] which is present on NCI-N892

Iodination and immunoreactivity. Albumin-free antibodies were labeled with ^{125}I by the chloramine-T method.[19] Briefly, 25 μL borate buffer (0.2 mol/L, pH 7.65) containing 0.9 mmol/L chloramine-T (Eastman Kodak Co, Rochester, NY) per millicurie of Na^{125}I (Amersham, Arlington Heights, Il; 13 to 17 mCi/μg) was used to start the reaction, and 25 μL borate buffer containing 20 mmol/L sodium metabisulphite (Sigma, St Louis) was used to quench the reaction. For immunoassays, a reaction time of 30 seconds was used with 90 μg T101 and 1 mCi Na^{125}I. For cytotoxicity studies, we used 90 μg T101 or 60 μg 9.2.27 and 5 to 6 mCi Na^{125}I and a reaction time of two minutes. Bound ^{125}I was separated from free ^{125}I by a Sephadex G-25M column (Pharmacia, Uppsala, Sweden), which was precoated with 1% bovine serum albumin (BSA) (Sigma) in phosphate-buffered saline (PBS) and extensively washed with borate buffer. The labeled protein, detected as a first peak by a shielded radiation monitor, was directed through a disposable tubing system (Namic, Hudson Falls, NY) into the product vial. Immunoprecipitation with 10% trichloroacetic acid generally yielded >95% precipitable radioactivity Radiolabeling and separation of bound from free ^{125}I were performed under sterile

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conditions at room temperature. The protein concentration of the product was determined in a spectrophotometer by the Bio-Rad assay (Bio-Rad Laboratories, Richmond, Calif), and the radioactivity was measured in a radioisotope calibrator (Capintech, Montvale, NJ).

The immunoreactivity of the radiolabeled antibodies was determined according to the method of Lindmo et al.[20]

Scatchard analysis and indirect immunofluorescence. Specific ^{125}I-T101 binding to HUT 102, MOLT-4, and HUT 78 cells was analyzed by the method of Scatchard.[21] In short, cells were washed twice in PBS (pH 7.4) and reconstituted in PBS + 1% BSA at 2 × 10^6 cells per milliliter. Cells in a volume of 200 μL in the absence or presence of excess unlabeled T101 were incubated with 100 μL ^{125}I-T101 in serial dilutions in microcentrifuge tubes (Bio Plas Inc, San Francisco), precoated with 1% BSA for two hours at 4 °C in a rotating rack. For determination of the total applied radioactivity, 75-μL samples (1x) were counted directly in a gamma counter (Beckman Instruments Inc, Irvine, Calif). To measure the cell-bound radioactivity, 75-μL samples (2x) were applied to 96-well filter plates (V&P Scientific Inc, San Diego), precoated with 1% BSA placed on a vacuum drain. The wells were washed eight times with PBS before counting the filters. Specific binding was determined by subtracting nonspecific binding from total binding measured for the same concentration of ^{125}I-T101. For calculation of the dissociation constant (K_d), the immunoreactivity of the conjugate was taken under consideration.

For indirect immunofluorescence, log-phase cells were washed twice in PBS and incubated with a saturating concentration of T101 or a purified mouse myeloma protein MOPC 21 (IgG_1; Litton Bionetics, Kensington, Md) for 30 minutes at 4 °C, washed three times in PBS, and incubated with saturating concentrations of purified fluorescein-conjugated goat antimouse IgG secondary antibody (Becton Dickinson, Mountain View, Calif). Stained cells (2 to 5 × 10^4) were analyzed in a fluorescence-activated cell sorter (Epics V; Coulter Electronics, Hialeah, Fla) using the 488-nm-wavelength beam (200 mW) and collecting fluorescent signals above 510 nm.

External irradiation. Cells in log-phase were washed twice in PBS to obtain a single cell suspension and reconstituted in RPMI 1640 in 15-mL tubes (Falcon; Becton Dickinson, Cockeysville, Md) at a cell density used for plating The tubes were placed in a Lucite block to ensure electron equilibrium and irradiated at room temperature using a 15-meV photon beam from a Varian 20 linear accelerator with a dose rate of 500 rad per minute. Thereafter, cells were seeded in triplicate in 35-mm plastic Petri dishes (Falcon) at a number producing at least 50 colonies (aggregates >50 cells in control plates) Culture medium consisted of RPMI 1640, supplemented with 10% FBS, penicillin 100 U/mL (GIBCO), streptomycin 100 μg/mL (GIBCO), L-glutamine 1 mmol/L (GIBCO), and 0.3% agarose (Sea Kem; FMC Corporation, Rockland, Me) using 2 mL as base layer and 1 mL with added cells as top layer. For estimation of surviving fractions at highest radiation doses, 100-mm Petri dishes (Falcon) were used in triplicate containing 28-mL base layer with 0.5% agarose and 7-mL top layer with 0.3% agarose and sevenfold the former cell number. All dishes were incubated in a well-humidified atmosphere of 5% CO_2–95% air, and colonies were counted with an inverted-phase microscope after two to three weeks.

Radioimmunotherapy. For each of three experiments, cells in log-phase were washed twice in PBS and divided in samples of 9 × 10^6 viable single cells. Viability was determined by trypan blue (Sigma) exclusion. Subsequently, cells were incubated with ^{125}I-T101 (high specific radioactivity) at the saturating concentration (for HUT 102, 2 μg/mL per 1 × 10^6 cells; for MOLT-4 and HUT 78, 0.5 μg/mL per 1 × 10^6 cells). Control samples were untreated cells, cells treated with a saturating concentration of unlabeled T101, cells treated with a similar protein concentration of the irrelevant antibody 9.2.27 bound to ^{125}I (high specific radioactivity), and cells treated with Na^{125}I at a radioactive dose similar to ^{125}I-T101. Incubation was performed in a well-humidified atmosphere of 5% CO_2–95% air for one hour at 37 °C. Thereafter, cells were washed twice in PBS and counted for cell-bound radioactivity in a radioisotope calibrator with results expressed in dpm per viable cell. For cells treated with Na^{125}I and washed afterward, again Na^{125}I was added at a dose similar to cell-bound ^{125}I-T101. For dose–response curves, additional samples were incubated with ^{125}I-T101 at various protein concentrations in the same way.

Prolongation of exposure time to radiation of ^{125}I was achieved under frozen conditions. In this respect, all samples were divided in six aliquots containing approximately 1 × 10^6 cells reconstituted in 1 mL RPMI 1640 supplemented with 80% FBS and 7.5% dimethyl sulfoxide (Rimso-100; Research Industries Corp, Salt Lake City) in cryotubes (Nunc, Intermed, Roskilde, Denmark) and stored in shielded containers after rapidly freezing at −70 °C.

For all experiments, one tube from each group was thawed at six time points. Cells were washed once in RPMI 1640 and 10% FBS, the viability was determined, and the cell number adjusted before plating. Surviving fractions for two cell densities were calculated from colony formation in soft agarose in 35-mm dishes performed in triplicate as described before.

In experiment I, HUT 102 and MOLT-4 cells either were incubated with a saturating concentration of ^{125}I-T101 (200 μCi/μg), with a saturating concentration of T101, with ^{125}I-9.2.27 (150 μCi/μg), or with additional Na^{125}I, or were control cells. The second experiment (II) was set up in the same way. ^{125}I-T101 (75 μCi/μg) was not only used at a saturation concentration, but 25% of this dose also was applied to another sample. The specific activity of ^{125}I-9.2.27 in this experiment was 80 μCi/μg. In experiment III, a dose–response effect for various protein doses of ^{125}I-T101 (55 μCi/μg) was further explored in HUT 102, MOLT-4, and HUT 78. Calculation of the cell-associated activity in the three experiments expressed in dpm per viable cell is shown in Table 1.

Estimation of internalization of ^{125}I-T101. Cells were washed twice in PBS, reconstituted in PBS + 1% BSA, divided in two aliquots, and subsequently incubated with a saturating concentration of ^{125}I-T101 in Eppendorf microcentrifuge tubes (Brinkmann Instruments Co, Westbury, NY) precoated with 1% BSA for two hours at 4 °C in a rotating rack or for two hours at 37 °C and stirred frequently. Thereafter, 75-μL aliquots served for either total applied radioactivity (1x), cell-bound radioactivity (4x), or cell-bound radioactivity after trypsinization (0.5 g trypsin 1:250 + 0.2 g EDTA/1; GIBCO) for ten minutes at room temperature (4x).

RESULTS

Iodination of monoclonal antibodies. The labeling of T101 to ^{125}I with a chloramine-T reaction time of 30 seconds yielded specific activities of 3.5 to 9 μCi/μg and immunoreactivity >80%. By prolongation of the reaction time to two minutes and use of a higher dose ^{125}I, we were able to increase the specific radioactivity without excessive loss of immunoreactivity. For example, when the specific activity of ^{125}I-T101 increased from 55 to 200 μCi/μg, the immunoreactivity decreased from 80% to 50%. Similarly, when the specific activities of ^{125}I-9.2.27 were increased from 80 to 155 μCi/μg, the immunoreactivity decreased from 70% to 40%.

Antigenic determinants of T65. From Scatchard analysis (Fig 1), the number of T65 sites per cell was calculated to be 350,000 for HUT 102 and 25,000 for both MOLT-4 and HUT 78 in log-phase growth. Nonspecific binding of ^{125}I varied with the viability of the cells and increased dramati-

Table 1. Cytotoxic Effects of Unlabeled T101, ^{125}I-T101 at Two Concentrations, ^{125}I-9.2.27, and Free Na^{125}I on HUT 102, MOLT-4, and HUT 78 Cells Under Frozen Conditions

			Experiment I				Experiment II				Experiment III			
				Survival Fraction (%)				Survival Fraction (%)				Survival Fraction (%)		
Cell Line	Drug	Dose	dpm/cell	Day 10	Day 20	Day 30	dpm/cell	Day 10	Day 20	Day 30	dpm/cell	Day 10	Day 20	Day 30
HUT 102	Untreated			100	100.0	100.0		100	100.0	100.0		100	100.0	100.0
	T101	Saturation		97	101.0	100.0		102	86.0	90 0				
	^{125}I-T101	Saturation	52.0	8	0.6	< 0.1	38.0	10	1.0	0.1	41.0	4	0.2	< 0.1
	^{125}I-T101	25% saturation					18.0	22	5 0	1 0	21.0	7	0.5	< 0.1
	^{125}I-9.2.27		8.0	56	32 0	17.0	16.0	57	36.0	19.0				
	Na^{125}I		62.0	86	58.0	38.0	28.0	70	38.0	16.0	24.0	76	48.0	31.0
MOLT-4	Untreated			100	100.0	100.0		100	100.0	100.0		100	100.0	100.0
	T101	Saturation		98	102.0	96.0		100	96.0	85.0				
	^{125}I-T101	Saturation	10.2	1	< 0.1	< 0.1	3.7	5	0.2	< 0.1	4 1	4	0 4	< 0.1
	^{125}I-T101	25% saturation					1.8	17	3.0	0.5	1 8	9	0.8	< 0.1
	^{125}I-9 2.27		2.8	80	64.0	58.0	3.5	90	80.0	73.0				
	Na^{125}I		12 6	59	15.0	3.0	3.8	55	24.0	11.0	4.7	75	35.0	16.0
HUT 78	Untreated											100	100.0	100.0
	^{125}I-T101	Saturation									3.1	51	32.0	19 0
	^{125}I-T101	25% saturation									1.2	70	50.0	35.0
	Na^{125}I										2.9	98	76.0	53.0

cally with concentrations higher than saturation in HUT 102 and MOLT-4. The K_d was 0.510 nmol/L, 0.106 nmol/L, and 0.223 nmol/L for HUT 102, MOLT-4, and HUT 78, respectively.

Comparison of indirect immunofluorescence intensity for T101 on the three cell lines confirmed the presence of the highest number of T65 sites on HUT 102 cells (Fig 2). Considering the difference in autofluorescence of control cells, MOLT-4 and HUT 78 showed similar fluorescent peaks for T101 binding. Of interest, a large fraction of HUT 78 cells were negative for T101 binding.

External irradiation survival. The in vitro x-ray radiation survival curves for the three T cell lines are shown in Fig 3. Decreasing surviving fractions with increasing irradiation doses demonstrated exponential lines without evidence of a shoulder region at low irradiation doses as expected for lymphoid cell populations. Radiation sensitivity differed extensively, with a D_0 of 255 rad for HUT 102 as the least sensitive cell line and a D_0 of 45 rad for MOLT-4 as most sensitive. HUT 78 cells had an intermediate sensitivity, with a D_0 of 100 rad.

Radioimmunotherapeutic studies. The survival of cells exposed to radiolabeled antibodies in vitro is shown in Figs 4 and 5 and Table 1. Experiments I and II included treatment of HUT 102 and MOLT-4 with saturating concentrations of ^{125}I-T101 or unlabeled T101, and with ^{125}I-9.2.27 or Na^{125}I. Unlabeled T101 had no effect on colony survival in soft agarose compared with untreated cells. Treatment with Na^{125}I and ^{125}I-9.2.27 seldom decreased survival to less than 10%, even after exposure times of 30 days. In contrast, ^{125}I-T101 had a marked cytotoxic effect with >2 logs' cell killing within three weeks. The cytotoxicity was highly dependent on the duration of exposure and was exponentially increasing with exposure time.

The cytotoxic effects of ^{125}I-T101 on MOLT-4 were similar to those on HUT 102, when the inherent radiosensitivity of each cell line is taken into consideration. The cell-bound radioactivity after incubation was remarkably lower for MOLT-4 cells because of the low number of binding sites. Nonetheless, colony survival in MOLT-4 was more reduced at each time point because of its greater radiosensitivity. Na^{125}I appeared to have a greater cytotoxic effect caused by this sensitivity, regardless of the fact that a lower amount of radioactivity was associated per cell.

The second experiment (Fig 4) was performed to examine the influence of specific activity and antibody concentration. While at first instance, ^{125}I-T101 at 200 μCi/μg was used, in this experiment, specific activity was 75 μCi/μg, which resulted in reduced cell-bound radioactivity. In both cell lines, colony survival was less affected. When tumor cells were exposed to 25% of the saturating concentration of antibody, the cell-bound radioactivity and cytotoxicity were reduced proportionally.

To delineate further the effects of decreasing amounts of cell-bound radioactivity, a third experiment (III) was performed with ^{125}I-T101 at varying antibody concentrations explored in HUT 102, MOLT-4, and HUT 78 (Fig 5). At saturating concentrations of ^{125}I-T101, resulting in 41, 4.1, and 3.1 dpm per cell, respectively, the first time point of the survival curve was determined immediately after incubation. Cytotoxicity was 34% in HUT 102 and 52% and 14%, respectively, in MOLT-4 and HUT 78. A higher concentration of ^{125}I-T101 in HUT 102 (57 dpm per cell) showed a slightly increased cytotoxic effect. Lower concentrations of the conjugate resulted in decreased cytotoxicity related to exposure time in all cell lines. In HUT 78 cells, the low activity of bound ^{125}I-T101 at saturating concentration indicated a low number of T65 determinants. Again, free Na^{125}I at radiation doses approaching the saturating concentration of bound ^{125}I-T101 showed minor cytotoxicity. Table 1 comprises a summary of surviving fractions (percentages) in the three experiments for various time points of exposure.

From survival curves obtained by ^{125}I-T101 at saturating concentrations, the D_0 dose to produce 37% surviving colonies was calculated. These doses were, for HUT 102, MOLT-4, and HUT 78, 25 × 10^4, 2.5 × 10^4, and 8 × 10^4

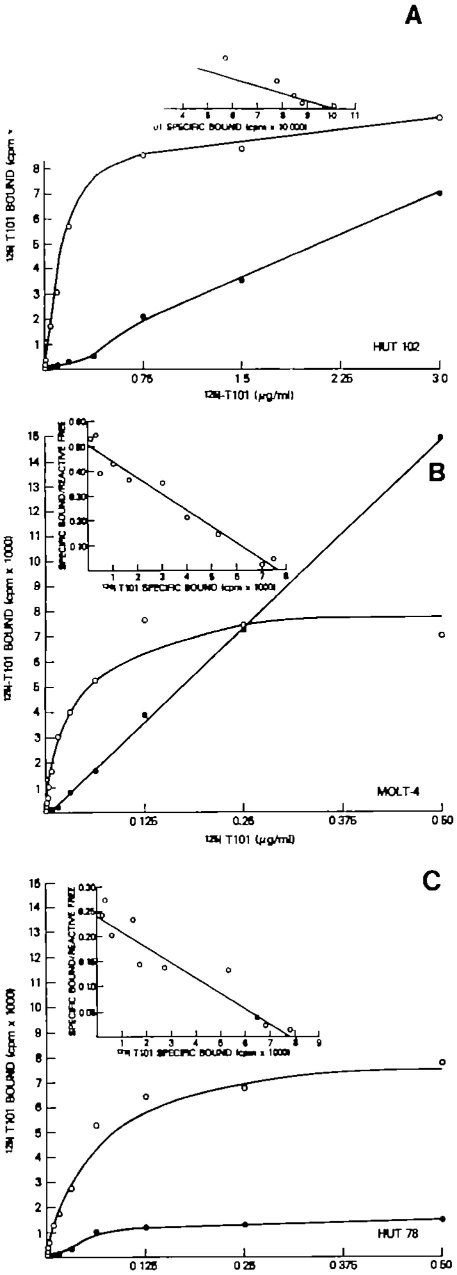

Fig 1. Scatchard analysis of ^{125}I-T101 binding to (A) HUT 102, (B) MOLT-4, and (C) HUT 78. Specific binding (○) was calculated by subtracting nonspecific binding (●) from total bound ^{125}I-T101 to a consistent number of cells. For HUT 102 (viability 92%), the number of sites per cell was 350,000, and both for MOLT-4 (viability 90%) and HUT 78 (viability 77%), 25,000. The K_d was 0.510, 0.106, and 0.223 nmol/L, respectively.

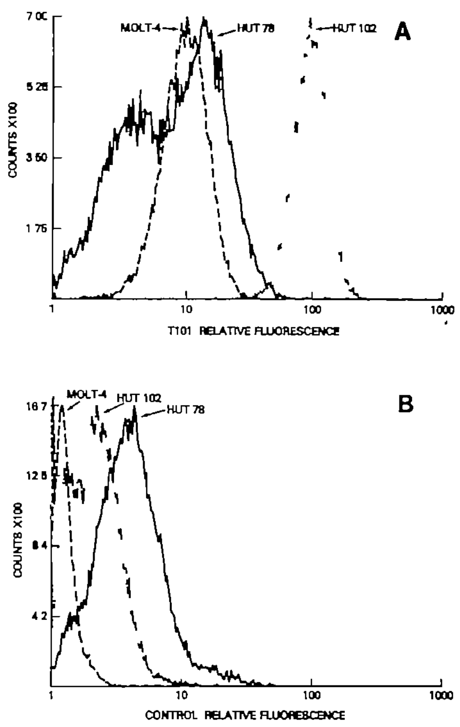

Fig 2. Indirect immunofluorescence of (A) T101 and (B) the control antibody MOPC 21 on HUT 102, MOLT-4, and HUT 78 cells.

decays per cell, respectively, demonstrating a similar radiosensitivity pattern to results obtained from external irradiation.

Internalization. Assuming that immunomodulation of an antigen–antibody complex after incubation at 4 °C is negligible, results of 37 °C and 4 °C of cell-associated ^{125}I-T101 were compared before and after trypsinization. After trypsinization, cell-associated activity was for HUT 102, 20%; MOLT-4, 0%; and HUT 78, 6.5% higher in cells incubated at 37 °C than in cells incubated at 4 °C. These differences indicate little internalization after two hours' incubation in vitro, while most ^{125}I-T101 is bound to the surface of the cells.

DISCUSSION

Radiolabeled monoclonal antibodies have the potential to produce tumor-specific cytotoxicity in vitro and in vivo. Studies to date suggest that most unlabeled monoclonal antibodies produce little cytotoxicity in vivo, even when they reach soft tissue tumor sites in saturating concentrations.[5,6] Our studies were designed to determine whether radiolabeled antibodies possess cytotoxic effects on reactive cells in vitro. We showed that human malignant cells expressing the T65 antigen could be killed specifically by ^{125}I-T101 of high specific radioactivity. This cytotoxicity was dependent on antigen density and radiation sensitivity of the cell line, the amount of cell-bound radioactivity, and the duration of radiation exposure. The cell-bound radioactivity was dependent on the antibody concentration applied for incubation, the specific activity of the immunoconjugate, and the antigen density of the target cell. Clinical trials with high doses of ^{125}I-T101 should be considered in T65-positive malignancies because of its extensive cytotoxicity in vitro and because of successful tumor targeting observed with ^{111}In-T101 in vivo.[11]

Monoclonal antibodies have opened a new modality for cancer treatment, since they offer a unique mechanism as

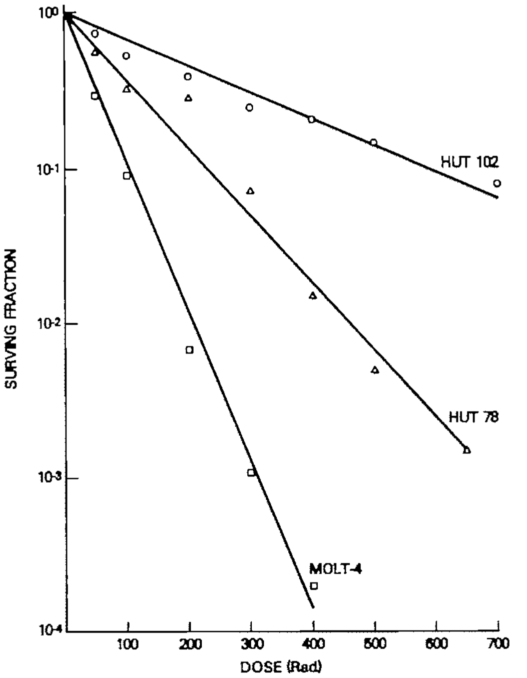

Fig 3. Survival curves for HUT 102 (○), MOLT-4 (□), and HUT 78 (△) after external irradiation at a dose rate of 500 rad per minute. The D_0 doses are 255, 45, and 100 rad, respectively.

specific carriers of therapy to tumor cells. Ideally, a radionuclide applied for this type of immunotherapy should have high linear energy transfer of a short range with little or no gamma radiation, a relatively short physical half-life, and reasonable chemical properties for radiolabeling. A radiolabeled antibody should retain sufficient immunoreactivity at high specific activity. Ionizing radiations from alpha or beta particles seem most appropriate for short-range damage.[22,23] In this respect, radioactive iodine has been studied mostly because of its well-known therapeutic application in thyroid disease and because it can be readily conjugated to many antibodies.

Thus far, the therapeutic application of radioactive iodine labeled to antibodies in patients has been limited to the use of ^{131}I.[12-14] The isotope has a physical half-life of eight days. Its medium-energy beta rays deliver short-range radiation at a high rate. However, ^{131}I also has a substantial amount of gamma ray emission, and perhaps not surprisingly, myelosuppression has been the dose-restricting factor. This toxicity should be considerably less with ^{125}I because it has only weak gamma rays. The decay of ^{125}I is by electron capture, followed by emission of a substantial number of Auger electrons with a highly efficient radiobiological effect within short range.[23] We chose to use ^{125}I, which appeared to be labeled easily to T101 at high specific activity with little loss of immunoreactivity. Because ^{125}I has a long half-life of 60 days and a low rate of radiation delivery, we decided to study the effects of continuous exposure in cells under frozen conditions. Cell samples treated with extracellular Na^{125}I or ^{125}I-9.2.27 as an irrelevant antibody were controls ^{125}I-T101 was highly cytotoxic to target cells at saturating concentration. The minor reduction in surviving fractions of control cells was most likely caused by weak gamma rays and nonspecific binding of ^{125}I.

In all three cell lines studied, ^{125}I-T101 cytotoxicity was dependent on dose (cell-bound radioactivity) and duration of exposure. This lethal effect is probably attributed to DNA strand breakage because the indirect action by free radicals and repair of sublethal damage are inhibited in the frozen state.[24] The radiation dose delivered originated from cell-bound radioactivity, which was determined by the antibody concentration, the specific activity of the immunoconjugate, and the number of cell surface receptors. We were able to achieve relatively high specific activities (55 to 200 μCi/μg) with acceptable immunoreactivity (80% to 50%). Using the same antibody preparations, the cell-bound radioactivity varied markedly between cell lines due to considerable differences in antigen density. For example, HUT 102 (3.5 × 10^5 sites per cell) had more cell-bound radioactivity than MOLT-4 (2.5 × 10^4 sites per cell) at saturation in each experiment (52 and 38 dpm per cell v 10.2 and 3.7 dpm per cell). The dose–response relationship for all cell lines in the

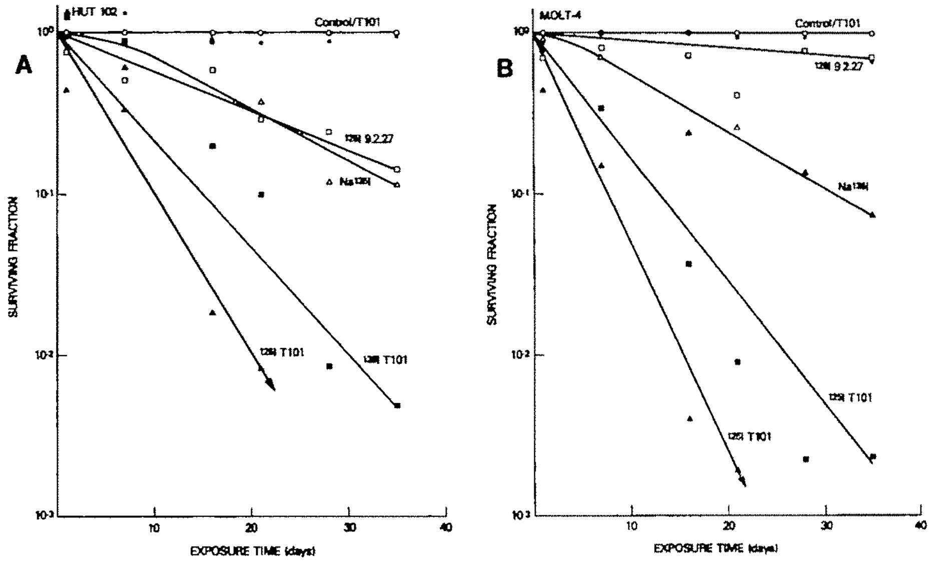

Fig 4. Experiment II survival curves of (A) HUT 102 and (B) MOLT-4 exposed under frozen conditions to T101 (●), ^{125}I-9.2.27 (□), ^{125}I-T101 at saturating concentration (▲), ^{125}I-T101 at 25% of the saturating concentration (■), and Na^{125}I (△); untreated cells (○). Radioactivity per cell in dpm is indicated in Table 1.

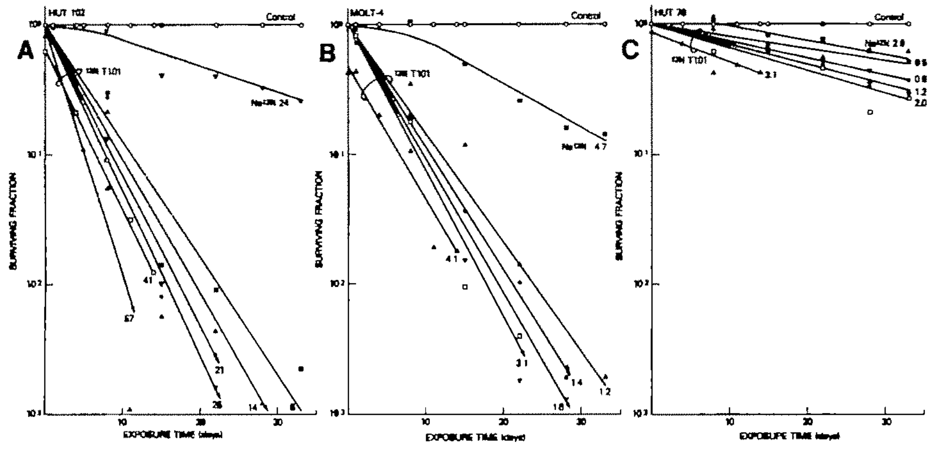

Fig 5. Experiment III survival curves of (A) HUT 102, (B) MOLT-4, and (C) HUT 78 exposed under frozen conditions to various concentrations of ^{125}I-T101 and free Na^{125}I. Radioactivity per cell is indicated in dpm in the figure. Symbols for HUT 102: ^{125}I-T101 at 200% of saturation (△), saturation (□), 50% (▽), 25% (●), 12.5% (▲), 6.25% (■), and Na^{125}I (▼). Symbols for MOLT-4 and HUT78: ^{125}I-T101 at saturation (△), 50% of saturation (□), 25% (▽), 12.5% (●), 6.25% (▲), and Na^{125}I (■). All untreated cells (○).

three experiments could be approximated by a straight line in the semilogarithmic plots. There was no shoulder on the curves, which is to be expected for lymphocytes (Fig 3) and for frozen cells that are inhibited to repair sublethal damage.

Despite the lymphoid cell origin of each of these cell lines, there were marked differences in radiosensitivity to both x-ray and ^{125}I irradiation. For external irradiation, MOLT-4 (D_0 45 rad) was more sensitive than HUT 78 (D_0 100 rad), which was more sensitive than HUT 102 (D_0 255 rad). These sensitivity patterns were similar after exposure to ^{125}I. For each cell line, the D_0 dose from ^{125}I at saturating concentration was approximately similar for immunoconjugates of high or low specific activity. For MOLT-4, the D_0 averaged 2.5×10^4 decays per cell; for HUT 78, 8×10^4 decays per cell; and for HUT 102, 25×10^4 decays per cell. Because of its greater inherent radiosensitivity, cytotoxicity was far greater for MOLT-4 than for HUT 102. Nonetheless, a radiolabeled relevant antibody can induce considerable toxicity in less radiosensitive cells, as shown for HUT 102. In the clinical situation, most T cell lymphomas are expected to be more radiosensitive than HUT 102, and to have less T65 receptor binding sites. Although saturating antibody concentrations can be reached in vivo, a 2 to 3 logs' cell killing was still achieved at less than saturating antibody concentrations within four weeks' exposure for both HUT 102 and MOLT-4. (Figs 4 and 5). In various experiments comparing the effect of x-ray radiation in frozen and live cells, the D_0 was four to five times higher under frozen conditions.[24,25] From these data one might expect even greater cytotoxicity of cell-bound ^{125}I in vivo. In previous studies of Warters et al,[26] ^{125}I was shown to be far more toxic to Chinese hamster ovary (CHO) cells when incorporated into DNA (^{125}I-iododeoxyuridine) than when bound to the cell membrane (^{125}I-concanavalin A) with a D_0 of 100 and 12,600 decays per cell, respectively. Although the D_0 for our cell lines was even higher than for membrane-bound ^{125}I in CHO cells, much of this difference can be ascribed to the frozen state of our cells.

In the experiments we observed little or no internalization of the T65 antigen after the one-hour incubation period. T65 modulation by T101 in vitro and in patients assayed by indirect immunofluorescence was shown to be a rapid process of a marked degree dependent on cell type, antibody concentration, exposure time, and temperature.[9] Much evidence exists that this modulation is caused by intracellular accumulation of the antigen–antibody complex.[10] Because in T cell lymphomas increased internalization may be expected on continuous infusion, ^{125}I-T101 would be predicted to have greater cytotoxicity in patients. To gain further knowledge on the potential therapeutic effect of ^{125}I-T101 in vivo, studies of the newly developed MOLT-4 xenograft model may be helpful.[27]

In order to achieve optimal therapeutic effects in patients, the administration of high doses of ^{125}I-T101, either continuously or at regular intervals, is probably necessary. Previous studies with monoclonal antibodies have encountered several problems, such as the development of endogenous antimouse antibodies.[5,14] These human antibodies effectively neutralize the effect of murine monoclonal antibodies, expressed in major changes in the biodistribution, a rapid clearance by the liver, and a marked reduction of binding to tumor cells. Also, high titers of antimouse antibodies may result in severe allergic reactions and should, therefore, be monitored regularly during treatment. Deiodination of labeled antibodies in the liver and kidneys renders the isotope free for uptake in the thyroid gland. To prevent this uptake, the patient should be given Lugol's iodine solution.[14]

This study demonstrates that ^{125}I can be readily conjugated to monoclonal antibody T101 to produce an immunoconjugate of high specific activity and sufficient immunoreactivity. The immunoconjugate achieves substantial cytotoxicity in malignant T cell lines in vitro, despite the frozen state of the cells and the low degree of internalization. Even greater cytotoxicity may be predicted in vivo. Comparable studies demonstrated the specificity of ^{125}I-labeled monoclonal antibodies admixing nonbinding cells.[25] Clinical studies with T101 alone in CLL and with ^{111}In-T101 in T cell lymphoma patients show antibody distribution and binding in all known sites of disease, although some nonspecific uptake in liver and spleen can be expected. Thus, clinical trials under proper precautions with high doses of ^{125}I-T101 should be considered in patients with T65-positive malignancies.

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The Effect of Isoferritins on Granulopoiesis

By G. Sala, M. Worwood, and A. Jacobs

The evidence for a regulatory role of acidic isoferritins on hemopoiesis is not entirely consistent with our knowledge of ferritin biochemistry, and no clear picture of this phenomeonon has emerged. In the present study, we have been unable to confirm a consistent effect of purified heart (acidic), spleen (basic), or serum (glycosylated) isoferritins on CFU-GM colony formation in vitro. Inhibition of colony formation by cell extracts or conditioned media does not relate to the presence of acidic isoferritins, nor is this effect neutralized by a monoclonal antibody to acidic isoferritins. The composition of ferritin preparations previously described as inhibitory to CFU-GM colony growth could not be confirmed, and they were not found to be predominantly acidic in nature. Our data do not support a role for acidic isoferritins as inhibitors of granulopoiesis.

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BROXMEYER ET AL[1] have demonstrated the presence of inhibitory activity directed against granulocyte-macrophage colony forming cells (CFU-GM) in the blood and marrow cells of leukemic patients. Inhibition was shown against CFU-GM in the S phase of the cell cycle expressing a high density of Ia-like antigen.[2] This activity was isolated and biochemically characterized as a glycosylated protein of molecular weight (mol wt) between 450,000 and 500,000, with an isoelectric point (pI) between pH 4.6 and 4.9.[3] Subsequently,[4] this leukemia-associated inhibitory activity (LIA) was identified as "acidic isoferritin" based on the fact that (a) LIA and acidic isoferritin have a similar mol wt and overlapping isoelectric points; (b) ferritin was found in LIA samples as determined by immunodiffusion using antiserum that recognizes H subunits of ferritin; (c) all acidic ferritin preparations tested manifested inhibitory activity of the LIA type; (d) the inhibitory activity present in LIA and ferritin preparations was inactivated by antiserum specific for acidic isoferritins and not by an antiserum against spleen ferritin; and (e) 90% to 95% of the total protein of LIA consisted of ferritin. Interestingly enough, this same paper showed that LIA did not elute from a concanavalin A (Con A)–Sepharose column with the two main protein peaks. Recent work from this group[5] refers to acidic isoferritin inhibitory activity (AIFIA).

The identification of LIA as the acidic isoferritin was not entirely compatible with available information on ferritin biochemistry.[6] Ferritin is present in all tissues and low concentrations in biological fluids.[7] Tissue ferritins have 24 subunits of two types, H (mol wt approximately 21,000) and L (mol wt approximately 19,000). The different isoferritins contain different proportions of these subunits. The more acidic isoferritins with a pI between 4.8 and 5.2 contain a high proportion of H subunits, and the more basic proteins with a pI between 5.3 and 5.8 contain a high proportion of L subunits. It is possible to raise antibodies specific for the acidic or basic isoferritins that show low cross reactivity.[8] Acidic isoferritins predominate in a variety of tissues, including heart, placenta, kidney, lymphocytes and monocytes,[9–11] and erythrocytes and erythroblasts.[12,13] Basic ferritins predominate in liver and spleen, the main iron storage organs.

Bognacki et al[3] have suggested that AIFIA is associated with a glycoprotein. Unfortunately, the evidence about glycosylation of ferritin is contradictory. Shinyo et al[14] found that preparations of horse spleen ferritin contained about 25 residues of hexose per molecule and less than ten residues of fucose. S.J. Cragg and M. Worwood found that human spleen ferritin contained less than 0.5% carbohydrate (unpublished observations), and Covell and Worwood[15] found small amounts of hexose in rabbit liver ferritin (only about five glucose residues per molecule—presumably due to contamination). In contrast, Lavoie et al[16] found more than 50 residues of glucosamine per molecule and more than 25 residues of galactosamine, glucose, and galactose, and Cynkin and Knowlton[17] found between 37 and 72 residues of glucose per molecule of spleen liver and heart ferritin from horse (perhaps indicating contamination of the preparations).

Serum contains isoferritins with a similar range of pIs to those of tissue ferritins, but 60% to 70% of the ferritin in normal serum binds to Con A-Sepharose[18] and neuraminidase treatment converts the more acidic isoferritins to more basic ones. Pure serum ferritin preparations show two main bands in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of apparent mol wt 19,000 and 23,000, the latter being glycosylated. This is referred to as a G subunit.[19] Serum ferritin preparations react with anti-spleen ferritin and not with antiheart ferritin.[8] Worwood et al[18] found that in extracts from heart, spleen, and liver, only 1% of ferritin bound to Con A-Sepharose, which is compatible with the small amount of carbohydrate found in spleen ferritin (unpublished observations, S.J. Cragg and M. Worwood). However, Ruggeri et al[20] have recently reported that up to 5% of ferritin in extracts of spleen or heart bound to Con A-Sepharose, and that this fraction was consistently richer in H subunits, as measured by immunoradiometric assay (IRMA) using monoclonal antiheart ferritin 2A4, than the unfractionated material. They also demonstrated,[20] by isoelectric focusing and immunoblotting, that the glycosylated fraction of serum ferritin contained a significant proportion of ferritin that bound to antibody 2A4. Despite these somewhat contradictory findings, it seems that only a small fraction of tissue ferritin preparations will bind to Con A.

Another enigma concerns the lack of correlation between presence of AIFIA and the type of isoferritin present in a cell. Broxmeyer et al[5,21] showed that normal monocytes and

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macrophages produce an inhibitory factor for CFU-GM, BFU-E, and CFU-GEMM that they identified with acidic isoferritins and suggested a specific role for these in the normal feedback regulation of hematopoiesis. Lymphocytes and granulocytes, on the other hand, did not appear to produce AIFIA, despite their synthesis of ferritins with a high H-L ratio.[22] Heart ferritin itself was found to be inhibitory. In long-term cultures of murine bone marrow, Oblon et al[23] found that production of AIFIA was associated with a nonadherent marrow cell population distinct from macrophages. Of the three cell lines K-562, HL-60, and U-937, only the inhibitory activity of HL-60 extracts was neutralized by antiacidic ferritin antibody[21] and was thus thought to contain AIFIA. However, K-562 has the greatest amount of H-reacting ferritin.[24] Dorner et al[25] showed that different HL-60 cell lines with similar levels of ferritin synthesis and H-L ratio differed by six orders of magnitude in inhibitory activity, although inhibition by liver ferritin preparations correlated with H-L ratio.[27]

The inhibitor postulated by Broxmeyer et al is a glycosylated protein that binds to Con A and is inactivated by periodate treatment. The evidence that this is an acidic isoferritin is indirect, and we felt that there was enough contradictory information to merit further investigation in order to establish whether well-characterized acidic isoferritins inhibit colony formation by CFU-GM. If this were so, we could identify and characterize the active isoferritin in biochemical terms.

MATERIALS AND METHODS

Culture Conditions for Human Bone Marrow Granulocyte-Macrophage Colony Formation

The agar culture system used in our laboratory is a slight modification of the method described by Nicola et al.[27] Bone marrow from sternum or femur was obtained with the fully informed consent of patients undergoing open heart surgery or hip replacement operations. Marrow fragments were collected in culture medium containing 50 IU of preservative-free heparin per milliliter. Bone fragments were removed by filtration through a cotton wool plug, and low-density mononuclear cells were prepared using lymphocyte separation medium (Flow Laboratories, Irvine, Scotland, UK). The isolated cell suspension was suspended at 5×10^6 to 10^7 cells per milliliter of RPMI 1640 (Flow) with 20% heat-inactivated fetal calf serum (HIFCS; GIBCO, Ltd, Cambridge, England, UK) and allowed to adhere to the bottom of 1005 Falcon Petri dishes (Becton Dickinson, Oxford, England) at 37 °C for two hours. The nonadherent population contained less than 4% of alpha-naphthyl acetate esterase-positive cells (kit supplied by Sigma Chemical Co, Ltd, Poole, England, UK). Low-density nonadherent bone marrow cells (10^5) were suspended in 1 mL of Dulbecco's modified Eagle's medium (GIBCO) with 3.7 g/L of sodium bicarbonate containing 10% to 20% FCS, 0.3% bacteriological agar (Oxoid Ltd, Baringstoke, England, UK), 10% of a source of colony-stimulating activity (CSA) as indicated in the tables and added to 35-mm Falcon Petri dishes containing 0.1 mL of the test substance or appropriate control. Cultures were incubated for seven days at 37 °C in a humidified incubator in 5% CO_2–air mixture. The same batch of FCS was used for all the experiments. It was not heat-treated except where otherwise stated.

The sources of CSA used were (a) human placental conditioned medium prepared as described by Schlunk and Schleyer[28] and subjected to two cycles of adsorption with an IgG-enriched fraction of a rabbit antiferritin serum coupled to cyanogen bromide-activated Sepharose (Pharmacia, Ltd, Milton Keynes, England, UK); (b) GCT cell line conditioned medium (GCTCM; GIBCO); and (c) 5637 cell line conditioned medium,[21] a gift from Dr H.E. Broxmeyer (H.E.B.).

Aggregates of more than 40 cells were scored as colonies except when GCTCM was used, at which time aggregates of more than ten cells were scored The CFU-GM culture conditions used in the experiments performed in H.E.B.'s laboratory have been described.[21] Aspirates from posterior iliac crest of normal paid volunteers were used, and the samples were processed at 4 °C. Total low-density mononuclear cells were plated.

Colony Formation of U-937

Cultures were carried out as described with slight modifications Briefly, 2 mL of the agar mixture containing 800 cells were plated on Petri dishes containing 0.2 mL of the test materials. No source of CSA was required. The incubation time was 11 to 14 days.

Cultured Cell Lines

The cell lines used in this study, K-562, HL-60, and U-937, were the ones described by Wyllie et al.[4] They were grown in RPMI 1640 supplemented with 10% HIFCS (30 minutes at 56 °C) and subcultured twice a week. Cells used for the preparation of conditioned medium were subcultured at 3×10^5 cells per milliliter on the day before they were used to produce conditioned medium. Viability, assessed by trypan blue exclusion, was always higher than 90%.

Measurements of Ferritin Concentration

These were carried out using a two-site IRMA with antibodies to human spleen and heart ferritins[8] and the monoclonal antibody 2-A4 specific for heart-reactive ferritins[29] donated by Dr. P. Arosio.

Additions to Cultures

The effect of the compounds or extracts detailed later on the proliferation of CFU-C was quantitated by counting colonies or aggregates for test and control samples and applying the following formula: $\%\Delta$ = percentage change = $(T-C)/C \times 100$, where C is the number of colonies for the control sample and T is the number of colonies for the treated sample. A negative value indicates that inhibition of growth has taken place.

Purified ferritin preparations. The samples of ferritin from spleen, heart I and II, and serum were prepared and characterized in our laboratory as described by Worwood,[30] Jones and Worwood,[8] and Cragg et al.[19] Heart II was subjected to heat treatment in the presence of 1 mmol/L phenylmethyl-sulphonyl fluoride (PMSF) (Sigma). The subunit composition was measured by the method of Cragg et al.[19] Comparison of protein and iron stains after PAGE and isoelectric focusing showed that the tissue ferritins were pure. In the case of serum ferritin, all the bands seen after protein staining are detectable (at lower concentrations) after immunoprecipitation. Partially purified preparations of heart and liver ferritin (ie, extracts) from iron-loaded human tissue rich in H and L subunits, respectively, were prepared in the presence of 0.5 mmol/L PMSF by heat treatment and ammonium sulfate precipitation. The different ferritin preparations were dialyzed against phosphate-buffered saline (PBS) (Oxoid) for 24 hours and sterilized by passing them through 0.45-μm Millipore filters (Millipore, Ltd, Crawley, England, UK). No losses occurred during filtration. To avoid losses during sterilization, the serum ferritin tested in H.E.B.'s laboratory was made up at a concentration of 5×10^{-8} mol/L in PBS with 1 mg/mL of purified bovine serum albumin (Behringwerke AG, Marburg, FRG) that was delipidated and deionized as described by Iscove et al.[31] All ferritin preparations were kept at 4 °C and final dilutions were made just before assay.

Acidic isoferritins prepared from the spleen of a patient with chronic myelogenous leukemia and a preparation of heart ferritin prepared by M. Dorner (heart III) were gifts from H.E.B. Their final concentrations were based on their quoted value of 0.1 mg of ferritin per milliliter.

Prostaglandin E_2. Prostaglandin E_2 (PGE_2; Upjohn, Ltd, Crawley, England, UK) was bought at a 10-mg/mL solution in absolute ethanol and stored at 4 °C. Dilutions in culture medium were made up before plating. Alcohol controls were used when appropriate, the maximum concentration being 0.1%.

Leukemic cell extracts. These were prepared according to the method of Broxmeyer et al.[1] Peripheral blood mononuclear cells (2 to 20 × 10^6/mL) from leukemic patients were suspended in RPMI 1640 and lysed by two cycles of rapid freezing and thawing. Lactic dehydrogenase measurements[33] indicated that more than 90% of the cells were disrupted. The mixture containing the disrupted cells was filtered through 0.45-μm Millipore filters (Millipore [UK] Ltd, Harrow, Middlesex, England) and stored in aliquots at −20 °C.

Conditioned media. The three cell lines mentioned earlier were cultured at an initial density of 10^6 cells per milliliter. The cell-free supernatant fluid (CM) was collected after three days following centrifugation (1,000 g for 15 minutes) and stored at −20 °C.

Complement-Mediated Cytotoxicity Assay

Complement-mediated cytotoxicity assay was performed as described by Broxmeyer [2]

Statistical Analysis

Three to five plates were scored for each sample. The probability of significant differences between samples was determined by use of the Student's unpaired *t* test. The results are expressed as %Δ ± SD %Δ. The standard deviation of %Δ is given by

$$SD_{\%\Delta} = \frac{T}{C}\sqrt{\frac{SD_C{}^2}{C^2} + \frac{SD_T{}^2}{T^2}} \times 100$$

where C and T were as defined, SD is the standard deviation (SD) of the %Δ, and SD_o and SD_T are the SD of the control and test, respectively.

RESULTS

Experiments carried out with human bone marrow. Bone marrow progenitors required the addition of a source of CSA to proliferate under the conditions of our assay. The concentration of heart (H)- and spleen (S)-reactive ferritin in these preparations are shown in Table 1.

Table 2 shows the inhibition of CFU-GM colony formation by ferritin preparations and cell extracts. The results are variable, but none of the pure ferritins significantly inhibited the proliferation of seven-day CFU-GM except for serum ferritin on one occasion only. In some experiments, serum and spleen ferritin appeared to show some degree of inhibition, but this never reached a significant level ($P < .05$). Heart ferritin preparations I and III showed no inhibitory activity. Similarly, ferritins prepared in the presence of PMSF were not inhibitory, indicating that absence of activity does not result from proteolysis.

Table 1. Concentration of Ferritin in Conditioned Media Measured by Assays With Monoclonal and Polyclonal Antibodies

CSA Source	Heart Ferritin (PoAb) M	Heart Ferritin (MoAb) M	Spleen Ferritin (PoAb) M
GCT CM	$<3 \times 10^{-12}$	5×10^{-12}	
HPCM (adsorbed)	$<3 \times 10^{-12}$	2×10^{-12}	
5637CM	$<3 \times 10^{-12}$	$<1 \times 10^{-12}$	

PoAb, polyclonal antibody; MoAb, monoclonal antibody.

Fresh leukemic cell extracts from eight patients were tested. Only one of these (W.D.) showed consistent inhibition of colony growth, but three other extracts showed inhibition with some marrow cultures but not others. Inhibition appeared to be less than the percentage of cells in S phase. CML spleen ferritin did not inhibit CFU-GM colony growth even when 32% of the progenitors were in S phase. The use of HIFCS did not affect the outcome. Human-CFU-GM were significantly inhibited by conditioned medium from U-937, K-562, and, to a lesser extent, HL-60. There was also significant inhibition by 10^{-6} mol/L PGE_2

Clusters were not counted in all experiments, but when the data were collected, the same trend as colony counts was shown.

Inhibition of U-937 colony formation. Broxmeyer et al[34] suggested that the human histiocytic cell line U-937 was a good target for the in vitro study of clonal growth regulation, and they found colony formation to be suppressed by lactoferrin, transferrin, and LIA. After our inability to demonstrate inhibitory activity in their CML spleen ferritin and heart ferritin III preparations using human bone marrow as a target, we used further aliquots of the same ferritin preparations in U-937 cultures. Cells were plated at different stages of their growth curve in order to vary the percentage in S phase, although in the event there was no significant difference in the S phase percentage except for experiment III d_3.

Both the preparations of CML spleen ferritin and heart III ferritin inhibited the proliferation of U-937 clonogenic cells, the former being possibly more active (Table 3). When the ferritin content of the samples was measured by IRMA, the heart-reactive ferritin present in the CML spleen ferritin preparation was 600 times lower than in the heart ferritin III (Table 4). These results suggest that there has been deterioration of the protein in these preparations and that their current inhibitory activity is not related to acidic isoferritin content.

Inhibition of CFU-GM colony formation in Indianapolis. Due to the difficulties encountered in trying to reproduce the results from Dr. Broxmeyer's group, one of us (G.S.) accepted his kind invitation to visit his Indianapolis laboratory to carry out additional studies.

Table 5 summarizes the results obtained there. In order to improve the sensitivity of the marrow target to inhibition by isoferritins, one set of plates was incubated in 5% O_2, 90% N_2, 5% CO_2 and the other in 95% air, 5% CO_2.[27] The number of colonies that developed at 5% O_2 was always higher, and

Table 2. Response of Human CFU-GM Growth to Different Additions to Culture

Additions		GCTCM % ± SD	HPCM Adsorbed % ± SD	5637 CM % ± SD
HtFn I	10^{-9} mol/L	0 ± 18, − 11 ± 9	+7 ± 35, +15 ± 5	−7 ± 7, −4 ± 22
HtFn III	10^{-9} mol/L			+10 ± 24‡, +21 ± 11*†
	10^{-10} mol/L			−3 ± 25‡, +17 ± 9*†
SpFn	10^{-9} mol/L	+1 ± 21, −3 ± 8, +6 ± 29	−20 ± 27, −21 ± 15, −11 ± 12	−16 ± 14, −13 ± 22
SeFn	10^{-9} mol/L	−35 ± 30	−16 ± 24, −15 ± 21	−27 ± 15,* −13 ± 30, +1 ± 19, −11 ± 23
CML SpFn	10^{-9} mol/L			−1 ± 19, +1 ± 19, 0 ± 17,‡ −3 ± 16*†
	10^{-10} mol/L			+2 ± 20, −7 ± 21, +4 ± 25‡
PMSF heart extract	2 × 10^{-9} mol/L	0 ± 33	+4 ± 27, +5 ± 30	+3 ± 18, −7 ± 12
PMSF liver extract	2 × 10^{-9} mol/L	−16 ± 29	−9 ± 24, 0 ± 30	+6 ± 19, −17 ± 14
Leukemic cell extracts	L.M.	−1 ± 13	−16 ± 10	
	W.D.	−27 ± 13§	−31 ± 10§	
	S.H.	−9 ± 11	−16 ± 10	
	N.W.		+17 ± 23	+12 ± 26
	A.B.		−9 ± 18	−15 ± 23, −15 ± 13§‖
	H C.		−2 ± 15	+9 ± 26, −14 ± 8§‖
	R.S.			−21 ± 13,* −19 ± 19
	R.W.			−10 ± 24
HL-60 CM		−36 ± 15§		−29 ± 27
K-562 CM				−83 ± 3§
U-937 CM	2%		−13 ± 13	
	5%		−71 ± 7,§ −74 ± 11§	−64 ± 11,§ −75 ± 10§
	10%		−99 ± 2§	
PGE_2	10^{-5} mol/L		−65 ± 5§	
	10^{-6} mol/L		−38 ± 9,§ −57 ± 15,§ −34 ± 21§	
	10^{-7} mol/L		−7 ± 9	

Additions to culture included ferritin preparations, cell extracts, conditioned media, and prostaglandin E_2, using three sources of CSA. Cells (10^5) were plated on each occasion. The results show the percentage of difference in clone numbers induced by each preparation. Control cultures with GCTCM alone yielded 258 ± 65 colonies plus clusters per 10^5 cells plated. The HPCM and 5637 CM yielded 135 ± 110[8] and 165 ± 91[8] colonies per 10^5 cells, respectively. For leukemic cell extracts, the equivalent of 2 to 4 × 10^5 leukemic cells were plated. Data from each column have not necessarily been collected using the same bone marrow. Subunit composition of the pure ferritins tested was as follows: heart ferritin (HtFn) I: 57%H, 43%L; II: 70%, 30%L; spleen ferritin (SpFn). 21%H, 79%L; serum ferritin (SeFn): 0%H, 75%L, 25%G.

*Percentage S phase = 15 ± 12.

†15% HIFCS was used.

‡Percentage S phase = 32 ± 10.§

§$P < .05$.

‖Percentage of S phase = 31 ± 8 §

there were more cells per clone than in plates grown in air. There was no difference, however, in the effect of added ferritins, and the results are analyzed without considering oxygen tension. There is no significant difference between the percentage of progenitors in S phase in the different experiments.

The preparation of CML spleen used at a concentration of 10^{-9} mol/L inhibited the proliferation of CFU-GM in five out of six experiments. The two preparations of heart ferritin, Ht I and Ht II, tested at concentrations as high as 5 × 10^{-9} mol/L failed to inhibit colony formation significantly, except on one isolated occasion when 10% inhibition was seen. Spleen ferritin (10^{-10} mol/L) produced up to 26% inhibition in some experiments, but this was not always seen at higher concentrations. Serum ferritin inhibited colony formation in most experiments, but pure bovine serum albumin used at the same concentration as that used in the serum ferritin dilution was also inhibitory in some tests, and it is difficult to interpret the ferritin results. When the different test substances were preincubated with monoclonal antibody 2A4 before their addition to the assay system (Table 6), no significant inactivation of inhibitory activity was found in any experiment. Although the degree of inhibition found with CML spleen ferritin was always less after preincubation with 2A4, this was not significantly different from the inhibition found without preincubation.

Ferritin synthesis by bone marrow cells in suspension culture. In order to determine the amount of ferritin generated and released into the medium on incubation by the target systems themselves, nonadherent bone marrow mononuclear cells were incubated in culture medium for periods up to 48 hours. Table 7 shows that 10^{-11} mol/L heart-type ferritin and 10^{-12} mol/L spleen-type ferritin are detectable after 18 hours.

Table 3. Effect of CML Spleen Ferritin and Heart Ferritin III on U-937 Colony Formation

		Experiment I		Experiment II	Experiment III		
Additions		d_1	d_2	d_1	d_1	d_2	d_3
CML SpFn	4×10^{-10}				−15 ± 11*	−20 ± 7*	−10 ± 9
	2×10^{-10}	−19 ± 10*	−41 ± 9*	−21 ± 7*			
	4×10^{-11}				−4 ± 11	−24 ± 2*	−7 ± 8
	2×10^{-11}	−9 ± 14	−35 ± 7*				
HtFn III	4×10^{-10}				−11 ± 9*	−14 ± 9*	+4 ± 13
	2×10^{-10}	−4 ± 10	−22 ± 6*	−14 ± 9*			
	4×10^{-11}				−10 ± 12	−12 ± 8*	−4 ± 13
	2×10^{-11}	−2 ± 10	−13 ± 13				
% S phase		25 ± 4	35 ± 11	38 ± 11	23 ± 10	24 ± 9	10 ± 14

The results are expressed as the percentage of difference from the control cultures (355 ± 46 cols/800 cells). The percentage of CFU-GM in S phase was determined by AraC suicide. d_1, d_2, d_3, indicate days after the last passage. SpFn, spleen ferritin, HtFn, heart ferritin.

*P < 05.

Ferritin content of ferritin preparations. Much of the evidence regarding the inhibitory activity of acidic isoferritins has been based on two preparations of CML spleen ferritin and heart ferritin.[4] Samples of these were kindly provided by H.E.B. for the present studies. Table 4 compares the ferritin content of these preparations with those made in this laboratory. In the cases of CML spleen ferritin and heart III ferritin supplied by H.E.B., the measured ferritin content is considerably less than the stated value in the case of CML spleen ferritin by almost three orders of magnitude. In addition, although these preparations were originally stated to consist predominantly of acidic isoferritins, the ratio of heart- to spleen-reactive ferritin is less than 1.0 in both cases. Heart I and II have heart-spleen ratios of 10.6 and 20.6, respectively.

DISCUSSION

The identification of LIA as an acidic isoferritin,[3,4] gives rise to several problems. The definition of AIF in this context appears to be based on a high ratio of H to L subunits although it is not clear why only H-rich isoferritins from some tissues, such as monocytes and myocardium, are inhibitory to CFU-GM, whereas others are not effective. AIF is stated to be inhibitory at a concentration of 10^{-18} mol/L,[4] a much lower concentration than those tested here and one that is not detectable by any available immunoassay system. Although normal monocytes and macrophages were considered to be the main source of AIF-regulating hemopoietic progenitors,[5] later experiments by the same group suggested that the major source was a nonadherent cell population distinct from macrophages.[23] In evaluating the inhibitory effect of cell extracts, HL-60 appeared to contain typical AIFIA[21], but Wyllie et al[24] showed that this ferritin has a low H subunit content. Conversely, K562 ferritin with a high H content has no AIFIA in the biological system.[21]

In order to try to resolve these and other discrepancies, we have examined the effect of well-characterized isoferritins on CFU-GM and U-937 colony growth in vitro. In addition, we have investigated ferritin preparations from H.E.B.'s laboratory and carried out studies in his laboratory.

We could find no consistent effect of either heart (acidic), spleen (basic), or serum (glycosylated) isoferritins on CFU-GM growth in vitro. Colony formation was, however, inhibited by conditioned medium from U-937, K-562, and HL-60, by 10^{-6} mol/L PGE_2 and by some leukemic cell extracts. These results and the others reported here suggest that there is no consistent effect of purified isoferritins on CFU-GM proliferation, although our other findings show that the target system used is sensitive to inhibition. Similar results were obtained in Indianapolis. Prior treatment of ferritin

Table 4. Protein, Heart, and Spleen Ferritin Concentration Measured in Preparations Used in Clonal Assays

		Ferritin Concentration (μg/mL)			Percentage Binding to Con A	
Ferritin Preparation	Protein* (μg/mL)	Spleen Assay (PoAb)	Heart Assay (MoAb)	Heart-Spleen Ratio	Spleen Assay	Heart Assay
Spleen	90	95.0	0 0	0		
Heart I	400	35 0	373.0	10 6	—	−1
Heart II	90	4.7	97.0	20.6	—†	8‡
Heart III	68 (100§)	22.2	18.8	0.85	11	12
CML spleen	<20 (100§)	0.147	0.028	0 19	9	—†
Serum	400	448.0	0.0	0	54	—†
Liver extract	514	456.0	0.0	0	6	—†
Heart extract	226	26.0	38.0	1.76	16	21

*See reference 32.

†Below limit of detection of assay.

‡After elution with 100 mmol/L a-D-methyl glucoside, 5% of the ferritin remained bound

§Concentrations quoted by H.E.B and used to calculate the molarity of ferritin used in the clonal assay.

Table 5. Response of Human CFU-GM to Different Ferritin Preparations at Atmospheric and Reduced Oxygen Tension

		Air + 5% CO_2			5% O_2–90% N_2–5% CO_2		
Additions		MI	MII	MIII	MI	MII	MIII
HtFn I	5×10^{-9} mol/L			+8 ± 16			0 ± 9
	10^{-9} mol/L	+2 ± 16	−11 ± 9		−15 ± 12	−24 ± 15	
	5×10^{-10} mol/L			−5 ± 22			−1 ± 11
	10^{-10} mol/L		−13 ± 15			−12 ± 14	
HtFn II	5×10^{-9} mol/L			−9 ± 8			+5 ± 14
	10^{-9} mol/L	−16 ± 10	−1 ± 17		−9 ± 17	−11 ± 12	
	5×10^{-10} mol/L			−10 ± 5*			−5 ± 14
	10^{-10} mol/L		−3 ± 11			−5 ± 13	
SpFn	5×10^{-9} mol/L			−9 ± 8			+1 ± 9
	10^{-10} mol/L	−5 ± 12	−30 ± 13*		−20 ± 8*	−24 ± 15	
	5×10^{-10} mol/L			+7 ± 11			−1 ± 8
	10^{-10} mol/L		−19 ± 10*			−26 ± 10*	
SeFn	10^{-9} mol/L			−28 ± 8*			+5 ± 12
	5×10^{-10} mol/L	−21 ± 9*	−30 ± 11*		−22 ± 7*	−26 ± 10*	
	10^{-10} mol/L			−19 ± 13*			−3 ± 15
	5×10^{-11} mol/L		−20 ± 6*			−26 ± 13*	
CML SpFn	10^{-9} mol/L	−7 ± 12	−23 ± 7*	−31 ± 6*	−23 ± 5*	−26 ± 9*	−23 ± 9*
	10^{-10} mol/L		−13 ± 10	−15 ± 6*		−10 ± 14	−6 ± 13
HtFn III	10^{-9} mol/L			−15 ± 6*			−8 ± 8
BSA control		+5 ± 12		−17 ± 8*	−25 ± 11*		−11 ± 9
% S phase		29 ± 14	23 ± 7	31 ± 16	20 ± 9		22 ± 11
% Ia positive		76 ± 3	69 ± 10	65 ± 5	80 ± 2		70 ± 5

Three target marrows were used, MI, MII, MIII. Results are expressed as percentage of difference from controls The number of CFU-GM in S phase have been estimated by ^{3}H TdR suicide and the number expressing Ia-like antigen by complement-mediated lysis with anti-Ia monoclonal antibody. 5637 conditioned medium was used HtFn, heart ferritin, SpFn, spleen ferritin; SeFn, serum ferritin; BSA, bovine serum albumin.

*Difference from control values $P < .05$.

Table 6. Effect of Ferritin Preparations on CFU-GM Growth in Two Marrows With and Without Preincubation With 2A4

	Air				5% O_2			
	MII		MIII		MII		MIII	
2A4	−	+	−	+	−	+	−	+
Control		−3 ± 10		+8 ± 13		+1 ± 20		+1 ± 1
HtFn I	−11 ± 9	0 ± 10	+8 ± 16	+1 ± 13	−24 ± 15	−9 ± 15	0 ± 9	−13 ± 10
HtFn II	−1 ± 17	0 ± 12	−9 ± 8	−9 ± 10	−11 ± 12	−19 ± 10	−5 ± 14	−10 ± 9
HtFn III	ND	ND	−15 ± 6*	−17 ± 14	ND	ND	−8 ± 8	−20 ± 8*
SpFn	−30 ± 13*	−11 ± 6	−9 ± 8	−2 ± 10	−24 ± 15	−26 ± 13*	+1 ± 9	−4 ± 18
SeFn	−30 ± 11*	−7 ± 4	−28 ± 8*	−17 ± 13	−26 ± 10*	−26 ± 13*	−5 ± 12	−11 ± 11
CML SpFn	−23 ± 7*	−16 ± 9	−31 ± 6*	−17 ± 14	−26 ± 9*	−10 ± 15	−23 ± 9*	−16 ± 12
BSA control	ND	ND	−17 ± 8*	−5 ± 10	ND	ND	−11 ± 9	−8 ± 11

MII and MIII are those tested in Table 5. 5637 conditioned medium was used. HtFn, heart ferritin; SpFn, spleen ferritin; SeFn, serum ferritin; BSA, bovine serum albumin; ND, not determined.

*Significant differences from control ($P < .05$)

Table 7. Ferritin Concentration in the Supernatant of Suspension Cultures of Nonadherent Bone Marrow Mononuclear Cells (10^6/mL)

	Incubation Time (h)	Spleen Assay PoAb (mol/L)	Heart Assay PoAb (mol/L)	Heart Assay MoAb (mol/L)
M1	18	2.4×10^{-12}	2.5×10^{-11}	1.2×10^{-11}
	42	4.2×10^{-12}	$2 7 \times 10^{-11}$	1.1×10^{-11}
	48	4.8×10^{-12}	2.5×10^{-11}	1.2×10^{-11}
M2	18	1.2×10^{-12}	3.0×10^{-11}	1.8×10^{-11}
	42	$3 6 \times 10^{-12}$	3.1×10^{-11}	1.6×10^{-11}
	48	3.6×10^{-12}	3.4×10^{-11}	1.7×10^{-11}

preparations with a monoclonal antibody specific to H-type ferritin did not significantly alter colony inhibition where this was present. We were unable to confirm either the ferritin content or H subunit content of the preparations supplied by H.E.B. although these showed some inhibitory effect on U-937 colony-forming cells. In addition to this, we have demonstrated the endogenous generation of acidic isoferritin within the CFU-GM target system.

We have no doubt regarding the inhibitory effect of some cell extracts but find little reason to relate this to their AIF content, especially as our own AIF preparations have no effect. The two preparations provided by H.E.B. appear to be inhibitory in some circumstances, but these do not contain predominantly acidic isoferritins. We suggest that the proposed regulatory role of AIF in hemopoiesis needs critical reassessment.

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HLA-Haploidentical Bone Marrow Transplantation for Severe Combined Immunodeficiency Using E Rosette Fractionation and Cyclosporine

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Eight patients with severe combined immunodeficiency received bone marrow cells from their HLA haplotype-identical fathers after bone marrow T cell depletion by rosetting with neuraminidase-treated sheep red cells. Because the method led to the infusion of a small percentage of T lymphocytes (0.1% to 0.3%), cyclosporin was given by continuous intravenous infusion for two months in order to prevent the occurrence of graft-v-host disease (GVHD). Three patients who did have residual nonfunctional T lymphocytes received busulfan and cyclophosphamide before transplantation. Engraftment was observed in seven patients, and severe GVHD was not seen. Two patients died early after the bone marrow transplantation because of prior infections, and a third died at day 90 from a B cell lymphoproliferative syndrome. The five other patients are doing well. Stable engraftment has been achieved with reconstitution of cell-mediated immunity in 5/5 and humoral immunity in 4/5 patients.

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TREATMENT OF severe combined immunodeficiency (SCID) relies on the engraftment of stem cells leading to normal development of T and B lymphocytes.[1] In this respect, HLA-matched bone marrow transplantation (BMT) has been used with success since 1968.[2] Until recently, in the absence of an HLA-matched donor, attempts at treatment through the use of fetal liver and thymus have been successful only in 10% to 25% of the patients.[3] In most cases, fetal tissue transplantation led to an incomplete immunological reconstitution occurring only after a 12- to 24-month period.[4] T cell depletion of HLA-unmatched bone marrow has therefore been developed in order to prevent graft-*v*-host disease (GVHD) and to allow a successful BMT across the HLA barrier, as suggested from animal models.[5] Two methods have been successfully used. Reisner et al[6-8] and other investigators[9-11] have described a technique of T cell removal by soybean lectin agglutination and rosette formation with sheep red cells that permits both a T cell and a B cell reconstitution in approximately 30% of patients, T cell reconstitution alone in 40% of the patients, while another 30% of patients died of preexisting infections in all but one of the cases. Two to three successive BMTs were required, however, to achieve durable engraftment in several patients. Reinherz et al[12] have used, with some success, a monoclonal anti-T cell antibody (anti-T12) in order to remove BM T cells. This method led to severe acute GVHD that has been cured by the repeated infusion of anti-T12.[12,13] We describe another method for the achievement of mismatched haploidentical BMT in patients with SCID that consists of rosetting with sheep erythrocytes (E) for BM T cell depletion, followed by in vivo prophylaxis of the GVHD by cyclosporine (CyA).[14]

PATIENTS AND METHODS

Methods

Bone marrow fractionation. Approximately 400 mL of bone marrow was taken from each donor by multiple aspirations of small volumes (<2 mL) under anesthesia. Mononuclear cells were prepared by a density-gradient separation on Ficoll-Hypaque. T lymphocytes were removed from the bone marrow cell suspensions by rosetting with neuraminidase-(Behringwerke, Marburg, FRG) treated sheep red cells[15] (1 unit of neuraminidase for 1 × 10^8 red cells). Cells that did not rosette were separated on a Ficoll-Hypaque gradient and, after washings, finally resuspended in RPMI 1640 medium (GIBCO, Glasgow, Scotland) The percentage of T lymphocytes pre- and post-depletion was assessed by immunofluorescence using the pan-T OKT3 monoclonal antibody[16] by enumerating ≥1,000 cells

Conditioning. The patients (No. 2, 4, and 6) with residual T cells received a conditioning regimen associating busulfan (8 to 16 mg/kg between day −9 and day −6) and cyclophosphamide (200 mg/kg between day −5 and day −2). In addition, patient No. 4, with Omenn's syndrome, received VP16-213[17] (600 mg/m² between day −15 and day −12).

CyA administration. A test dose of 4 mg/kg was given intravenously (IV) a few days before the BMT in order to calculate the daily dose to be given by continuous IV infusion to achieve a plasma level of 100 ng/mL. The treatment was started at day −1 and continued until day +60 except in patient No 4, who received CyA IV for three months and then orally for another three months. The CyA plasma level was checked twice a week and the dose adjusted depending on the plasma level and the daily surveillance of blood urea nitrogen and creatinine levels

Immunological analysis. T and B lymphocyte populations were determined by immunofluorescence using either specific monoclonal antibodies (OKT3, OKT4, OKT6, OKT8, OKT10, and OKT11) or polyvalent antihuman immunoglobulin (Ig) F(ab')₂ antiserum as previously described.[18] In vitro lymphocyte responses to mitogens, antigens, and allogeneic cells were assayed by standard microtiter plate assays and quantitated by ^{3}H thymidine incorporation.[18] Natural killer (NK) and T-dependent cytotoxicity tests to allogeneic targets were carried out as previously described.[19] Immunoglobulins G, A, M, and E were quantitated by nephelometry or an enzyme immunoassay Antibodies to blood group substances (isohemagglutinins), diphtheria and tetanus toxoids, and polio and influenza viruses were quantitated by standard serologic techniques. All patients were immunized subcutaneously with diphtheria and tetanus toxoids and polio virus (Institut Pasteur, Paris) on three or four occasions in a six- to eight-week period between day +90 and day +180 after BMT. They were sensitized to influenza virus

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(Institut Merieux, Lyon, France) twice during the same period. Ig preparations were given to the patients for a maximum of 90 days after the transplant. Serum antibody concentrations were thus measured at least three months after cessation of Ig therapy. Typical chromosome Y fluorescence was assessed with quinacrine analyses using standard techniques, as were karyotypic analyses. Ig allotype studies were performed by indirect hemagglutination using specific antisera (Dr L. Rivat, Bois-Guillaume, France).[20]

Evaluation of GVHD. GVHD was evaluated according to the clinical criteria of Glucksberg et al,[21] together with skin or rectum biopsy sample histologic examinations.

Patients

Table 1 gives the main data about the eight patients. These eight patients (aged 3 to 42 months, seven of them under age 1 year) were referred to the Hôpital des Enfants Malades for SCID. Three of them (No. 5, 7, and 8) had SCID characterized by a complete absence of T lymphocytes but a normal number of B lymphocytes[18]; two others (No. 1 and 3) were associated with adenosine deaminase (ADA) deficiency, and the remaining three patients (No. 2, 4, and 6) presented with nonfunctional T lymphocytes, one of them with Omenn's reticuloendotheliosis syndrome.[17] Patients No. 1, 3, 5, 6, 7, and 8 had no NK activity before transplant, whereas patients No. 2 and 4 exhibited normal or enhanced NK activity. The clinical status before BMT was excellent for two patients (No. 5 and 6), while the six others suffered from severe infections: protracted diarrhea in six, interstitial pneumonitis in four, sclerosing cholangitis in one, and bacille Calmette-Guérin sepsis in one. None of these patients had any evidence of maternofetal GVHD. Before BMT, the patients received appropriate anti-infectious therapy, intestinal decontamination by nonabsorbable antibiotics, ketoconazole, and IV immunoglobulins. They were protected in a Trexler's isolator (La Calhène, France). Because there were no matched siblings, the HLA-haploidentical father was chosen as BM donor. As shown in Table 2, for one of them, there was an HLA-A, B identity with the recipient. All others had a full haplotype HLA-A, B, DR antigen mismatched with the donor. The mixed leukocyte reaction (MLR) between donor and recipient was positive, >10,000 cpm above the background response in all cases. All patients' parents were advised of procedures and attendant risks in accordance with institutional guidelines and gave informed consent.

Table 1. Diagnosis and Clinical Status Before BMT

Patient No.	Age (mo)	Sex	Type of SCID	Clinical Condition	BMT Preparation
1	6	M	ADA deficiency	Interstitial pneumonitis, protracted diarrhea	0 then Bu, Cy*
2	42	M	Present but nonfunctional T cells	Interstitial pneumonitis (CMV), erythroblastopenia, protracted diarrhea, sclerosing cholangitis	Bu, Cy
3	3	F	ADA deficiency	Protracted diarrhea, interstitial pneumonitis, + meningitis (Asp)	0
4	4	F	Omenn syndrome	Protracted diarrhea, hepatosplenomegaly, erythroderma	VP16, Bu, Cy
5	4	M	Absence of T cells	Good	0
6	10	M	Present but nonfunctional T cells	Good	Bu, Cy
7	6	F	Absence of T cells	Protracted diarrhea, interstitial pneumonitis (Asp, P C.), bacille Calmette-Guérin sepsis	0
8	5	M	Absence of T cells	Protracted diarrhea	0

VP16, epipodophyllotoxin; Bu, busulfan; Cy, cyclophosphamide, Asp, aspergillus; P.C., *Pneumocystis carinii;* CMV, cytomegalovirus

*Two successive BMTs were performed.

RESULTS

T cell depletion. The percentage of T lymphocytes among harvested cells ranged from 8.8% to 21.5% (mean, 14%), as enumerated by immunofluorescence using the monoclonal antibody OKT3 (Table 3). In all cases, the percentage of infused T (OKT3$^+$) lymphocytes was <0.3% (<0.1% in seven cases). Patients No. 5 and 7 received BM cells containing 0.26% and 0.3% T3 (+) lymphocytes, respectively. The number of infused nucleated cells ranged from 0.3 to 2.0 × 10^8/kg. The yield of CFU-GM infused varied from 30% to 80%.

Engraftment. In patients conditioned before BMT, the hematologic reconstitution was rapid because neutrophils were above 500/µL within 20 days and platelets >50,000/µL within 40 days. In patient No. 4, the engraftment of polymorphs was documented by determination of the percentage of Y chromosome-positive cells by quinacrine fluorescence. In patients No. 2, 3, 4, 5, 6, 7, and 8, engraftment of lymphocytes was proven by HLA typing (patients No. 4, 5, 6, 7, and 8), by Y chromosome fluorescence and karyotype (patients No. 3, 4, and 7), and by ADA enzymatic activity

Table 2. HLA Typing

	Donor	Patient	MLR (Δ cpm × 10^{-3}) D→R*, C→R*	
1	A25 B27 DR3 A2 B35 DR4	A25 B27 DR3 A11 B9 DR3	14	24 5
2	A2 B15 DR7 A19-2 B18 DR2	A2 B15 DR7 A24 B7 DR3	18.	42.
3	A9 B7 DR2 A3 B39 DR1	A9 B7 DR2 A11 B22 DR6	24.5	33
4	A2 B5 DR7 A28 B12 DR5	A2 B5 DR7 A28 D12 DR3	20	74
5	A26 B22 DR1 A19-2 B13 DR2	A26 B22 DR1 A1 B8 DR4	46	28.5
6	A24 B35 DR5 A1 B8 DR3	A24 B35 DR5 A2 B8 DR7	76	48
7	A1 B8 DR7 A26 B13 DR4	A1 B8 DR7 A2 B21 DR1	10	19
8	A19-2 B35 DR6 A2 B38 DR4	A19-2 B35 DR6 A24 B18 DR5	19 5	19.5

Results are expressed as the difference between the ^{3}H thymidine uptake (cpm) in stimulated cultures minus background cpm (D→D*) (C→C*) MLR, mixed lymphocyte reaction; D, donor PBM; R*, 4,000-rad irradiated recipient PBM; C, unrelated control PBM

Table 3. T Cell Depletion of Bone Marrow

	Range	Mean
Number of harvested cells	53 to 131 × 10^8	82 × 10^8
Number of BM mononucleated cells	13 to 100 × 10^8	44 × 10^8
Total infused cells	1 6 to 15.0 × 10^8	6.2 × 10^8
Infused cells per kilogram	0.3 to 2.0 × 10^8	0.77 × 10^8
Percentage of T lymphocytes before depletion	8 8 to 21.5	14.0
Percentage of T3 lymphocytes infused	0.1 to 0.3	0.14

Values expressed represent the results of nine procedures

(patient No. 3) on blood samples (Table 4). A careful analysis by HLA typing of separated cells performed six months postgrafting and repeated one year thereafter showed not only the presence of T lymphocytes, but also a low, albeit significant, percentage (25%) of B lymphocytes and monocytes from donor origin in patients No. 5, 6, and 7. This proportion was evaluated by Y chromosome quinacrine fluorescence in patient No. 7 and by HLA typing of separated cells. The latter test gave one (+) positivity using antisera to donor HLA antigens, which is indicative of around 25% positive cells. In contrast, neither monocytes nor B lymphocytes of donor origin could be detected in patient No. 8. All B lymphocytes and most of the monocytes were of donor origin in patient No. 4, who received a high-dose conditioning regimen. In only one patient did a failure of engraftment lead to a second BMT after conditioning. The patient eventually died of infection, but the first manifestations of reconstitution were observed by ADA enzymatic activity determination in lymphocytes.

Graft-v-host disease. A mild GVHD (grade 1 in two patients, grade 2 in one patient) occurred between the third and fourth week after BMT in three patients (No. 6, 7, and 8) but resolved within 15 days after an increase in CyA dosage or steroid administration, or both. In two of them, GVHD occurred while the plasma CyA level was low (<30 ng/mL). No GVHD was seen in the five other patients. After CyA had been stopped for periods ranging from 11 to 16 months in five patients, no GVHD occurred. No signs of the capillary leak syndrome known to be facilitated by CyA had been observed.[22]

Table 4. Chimerism Studies Posthaploidentical BMT

Patient No.	ADA Activity	T Lymphocytes	HLA Typing	B Lymphocytes + Monocytes	Y Chromosome Fluorescence
2		+			
3	+	+			+
4		+		+ (>50%)	+
5		+		+ (25%)	
6		+		+ (50%)	
7		+		+ (25%)	+
8		+		−	

Chimerism analysis was performed 30 days postgrafting in patients No. 2 and 3 (ADA activity), 60 days postgrafting for patient No. 3 (HLA typing and Y chromosome fluorescence), 60 to 120 days, 180 days, and one year postgrafting for patients No. 4 through 8. Similar results were found 180 days and one year postgrafting and are shown. The figures shown in brackets are an evaluation of the percentage of donor-derived B lymphoctes and monocytes. In all cases, T lymphocytes were of donor origin only

Outcome. Two patients (No. 1 and 2) died early after BMT (days 35 and 50, respectively) of interstitial pneumonitis that was present before BMT. One patient (No. 3) died at day 95 of a polyclonal B lymphocyte proliferative syndrome proven to be of donor origin by karyotype while there was partial T and B cell function development. This syndrome was characterized by fever, coma, splenomegaly, lung infiltration, acidosis, and renal failure. Postmortem examination showed a diffuse infiltration by lymphoblasts containing cytoplasmic IgM (90% of cells), IgG or IgA (10%) with either λ chain (90%) or κ chain (10%). An IgM λ M component (10 to 12 g/L) could be detected in the patient's serum. No evidence of Epstein-Barr virus infection or other viral diseases had been found (negative serology and no EBNA antigen detected by immunofluorescence in the B lymphoblasts). The B lymphoblasts were of donor (male) origin as detected by Y chromosome quinacrine fluorescence, while the recipient was a female. The five patients alive are doing well and are without any signs of infection. They are home 705 to 975 days post-BMT.

Immunological reconstitution. Five patients (No. 4, 5, 6, 7, and 8) have experienced full cellular and significant humoral immunological reconstitution within four to six months after BMT (Table 4), as shown for patient No. 5 in Fig 1. This includes normal T and B lymphocyte numbers, positive proliferative responses to mitogens, antigens, and allogeneic cells, as well as positive skin tests to vaccination antigens and presence of serum IgG and IgM and of antibodies after vaccination. The IgA level remains low or absent in four of the patients. The serum IgG level is low in patients No. 5 and 8 despite the detection of some antibody responses (Table 5). Serum IgG and antibody responses detected in patient No. 8 six months posttransplant eventually disappeared, while the humoral immunity has remained present without alteration 12 to 18 months thereafter in the four

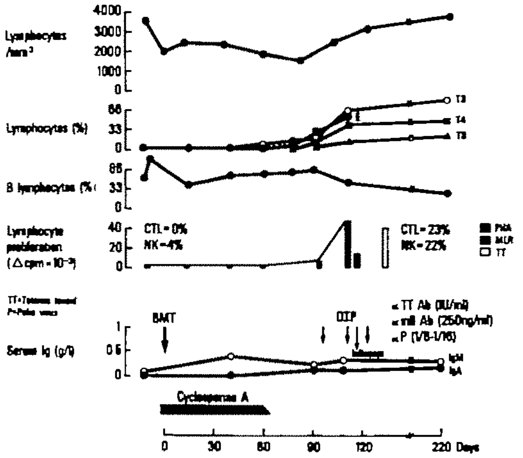

Fig 1. Bone marrow transplantation of patient No. 5. Immunological reconstitution. D.T.P. stands for diphtheria, tetanus toxoids, and polio virus immunizations. Full immunological reconstitution is still detected two years posttransplant.

Table 5. Immune Status Before and Six Months After Transplantation

	Patient 4		Patient 5		Patient 6		Patient 7		Patient 8	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Lymphocyte (counts/μL)	6,000	4,200	1,600	3,800	1,500	1,700	280	1,750	1,300	1,500
T3 %	74	68	0	78	47	62	0	65	0	49
E+ %	79	61	0	75	2	62	0	71	0	51
Lymphocyte transformation (cpm × 10^{-3})										
PHA	0.7	63	0.4	49	0.3	33	1.2	35.0	0.2	16
Allogeneic cells	1.0	142	0.5	47	0.7	18	0.3	12.0	1.1	33
Tetanus toxoid	0.5	50	ND	45	0.8	62	ND	16.5	1.2	12
Influenza	ND	ND	ND	8	ND	86	ND	7.0	ND	ND
48-h skin test to tetanus toxoid (>5-mm in duration)	–	+	–	+	–	+	–	+	–	+
B (sIg) %	0	4	62	20	36	10	73	13	77	30
Serum immunoglobulins (g/L),										
IgG	.35	5.31	12.7*	1.74	9.27*	5.74	0.70	7.18	.96	2.83
IgA	<.03	0.34	<0.03	0.08	<0.03	0.08	0.19	<0.03	<.03	<0.08
IgM	<.03	0.65	0.07	0.50	<0.03	2.70	1.92	2.81	.07	0.34
Isohemagglutinins anti-A, anti-B (titer: 10^{-1})	1:1	64:32	1:1	2:1	1	2	1	ND	1	ND
Antibody to:										
Polio virus (titer: 10^{-1})	ND	512	ND	16	ND	16.0	0	64	0	16.0
Tetanus toxoid (IU/mL)	ND	1	ND	1	ND	0.1	0	1	0	0.1
Diphtheria toxoid (IU/mL)	ND	ND	ND	ND	ND	5.0	ND	ND	0	ND
Influenza A (ng/mL)	ND	1,200	ND	250	ND	500.0	0	1,100	ND	200

Patients No. 6 and 7 were also found to have normal natural killer and T cell-mediated cytotoxic activities six months after BMT. Patient No. 7, who suffered from bacille Calmette-Guerin sepsis had a positive proliferative response and skin test to tuberculine when tested eight months post-BMT. Antibodies detected six months posttransplant in patient No. 8, as well as IgG, were no more evident six months thereafter ND, not done.

*Maternal IgG.

other patients. The serum IgM level is still high in patient No. 8 (2.5 to 3.0 g/L). Ig allotype studies performed in patients No. 5, 6, and 7 showed no difference between donor Ig allotypes and posttransplant recipient Ig allotypes. Ig allotypes could not be determined before transplant in these patients because they had either maternal IgG (No. 5 and 6) or no serum IgG (No. 7). T and B lymphocytes were detectable in patients No. 1, 2, 3, 4, 7, and 8 as early as one month after BMT, whereas T lymphocytes were detected only after day 70 in patients No. 5 and 6.

It is remarkable that the T4-T8 cell ratio was above 1 in five of six patients evaluable in contrast with data obtained in HLA-matched BMT[2] for patient No. 5 (Fig 1). There is no obvious explanation for this phenomenon.

DISCUSSION

The results reported here indicate the achievement of haploidentical BMT for SCID despite the infusion of a small number of T lymphocytes escaping the T cell-depletion procedure. Other methods have previously been used to achieve haploidentical BMT. BM fractionations using T cell agglutination by the soybean lectin and E rosetting methods led to T cell-free, stem cell-enriched preparation able to cure patients with SCID.[6,11] A failure of engraftment is sometimes observed. In one report, seven of 11 patients required two to three grafts.[6,7] Most patients experienced failure of B cell engraftment and a lack of donor T helper-host B cell cooperation, resulting in a persistent defect of humoral immunity.[7–9,11] The use of monoclonal antibodies (OKT3 or anti-T12) did not achieve a complete depletion of T cells, and severe GVHD was observed that was either lethal[23] or cured by the use of anti-T12 in vivo.[10,12] In addition, conditioning was always necessary in order to achieve engraftment.[12,13]

The combination of an incomplete T cell depletion by E rosetting followed by a short-term administration of CyA is a simple, reproducible method that gives a good yield of BM nucleated cells. We failed to achieve engraftment in only one patient with ADA deficiency, a condition known to be associated with resistence to reconstitution.[7,8] The use of the E rosetting procedure alone may spare some accessory cells required for engraftment that are depleted by the lectin separation procedure. Despite the administration of a low number of mature T lymphocytes, no severe GVHD occurred, and the moderate GVHD observed in three patients was transient and never occurred again. Finally, some B cell reconstitution, including antibody responses, was achieved in five patients, three of them having not received any conditioning. HLA typing post-BMT in the three latter patients indicates the predominance of host-type B lymphocytes, although a low percentage of B cells and monocytes are of donor origin for two of them. IgG allotype determinations are compatible with a donor origin of serum Ig found in three patients. Because the recipients' Ig allotypes could not be identified, one cannot rule out a possible production of Ig by host B cells, as previously shown following some, but not all, HLA-matched BMTs.[18] The transient presence of antibodies after immunization in one patient could be due to either a response of undetected engrafted mature B cells that eventually died or, less likely, a short, reversible activation of host B cells. That B cell functions were not persistently observed in the sole patient with no detectable B cell engraftment strongly argues for the donor B cell origin of humoral immunity in these patients, as already suggested by O'Reilly et al.[24] The question remains whether the observation of B cell reconstitution in four patients is fortuitous or secondary to the use of the method.

It is dubious whether the T cell-depletion technique using the E rosette method is responsible for an improvement in B cell function development. To a minor extent, this procedure differs from combining lectin agglutination and E rosetting, which leads to only a 40% B cell function. It can be envisaged that CyA might play a role in helping the B cell differentiation of donor stem cells. Such an effect could be mediated through a blockade of putative donor origin suppressor T lymphocytes, although these cells are only weakly sensitive to CyA,[25] or of host origin T or non T cells able to inhibit such a differentiation. The use of CyA has recently been outlined in preliminary observations of HLA-mismatched BMT in which the frequency of failure of engraftment was reduced by the administration of CyA to the recipient before or after the transplantation.[26,27] Such data are compatible with the low incidence of failure of engraftment in our patients (1/9 transplants).

The use of CyA could be hazardous, as illustrated by the occurrence in one patient of a B cell lymphoproliferative syndrome 2½ months after the BMT, while the patient received CyA for two months. Such syndromes have been observed in patients with SCID in the absence of any treatment or after thymic epithelial cell infusion[28,29] and kidney transplantation followed by CyA therapy.[30] The incidence of these B cell lymphomas has been found to be higher after T cell-depleted BMT,[31,32] even in the absence of CyA therapy. It is thus impossible to conclude for the respective roles of the underlying disease, of the absence of mature T cells after BM T cell depletion and of CyA administration in this B cell lymphoproliferative syndrome.

The treatment of more patients should yield additional information about the exact incidence of those lymphomas and about the usefulness of BM B cell—in addition to T cell—depletion. Although we are dealing with very young patients, the use of CyA was shown to be feasible, provided a plasma level around 100 ng/mL was maintained. Furthermore, in a comparative study, CyA has been shown not to delay the immunological reconstitution after HLA-matched BMT.[33]

Based on experimental data, several groups have suggested conditioning every patient requiring unmatched BMT, regardless of the type of SCID.[7,9,12,34] This method, however, increases the risk of life-threatening infections in patients with SCID. These patients often tend to be infected before the BMT. We decided to condition only those patients with residual, nonfunctional T cells who run a higher risk of engraftment failure. The conditioning used has been shown previously to be relatively safe at an early age and to permit reconstitution in patients with inherited diseases.[19]

In conclusion, it appears that our data are sufficiently promising to warrant further attempts at haploidentical BMT using E rosetting and a short period of in vivo CyA administration. This approach can be applied to SCID and to other inherited diseases of the immune system, such as Wiskott-Aldrich syndrome and lethal phagocytic cell dysfunctions.

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The Inhibitory Effect of Plasma Fibronectin on Collagen-Induced Platelet Aggregation

By D.G. Moon, J.E. Kaplan, and J.E. Mazurkewicz

Plasma fibronectin (Fn) has been proposed to have an antithrombotic effect, protecting against platelet and fibrinogen consumption after injury. The current study was designed to determine the effect of plasma fibronectin on collagen-induced platelet aggregation. In vitro aggregometry using an isolated homologous rat system, demonstrated a significant ($P < .05$) inhibitory effect of 120 μg/mL Fn on platelet aggregation as induced by 60 μg/mL fibrillar collagen (type I). The inhibition was evidenced by a threefold increase in lag time and a significant decrease in the rate and extent of aggregation. The hypothesis was also tested using an in vivo model of collagen-induced platelet aggregation. The model used was intravenous injection of 2 mg/kg of homologous type I collagen into anesthetized Sprague-Dawley rats. Injection of collagen preincubated with 4 mg/kg Fn resulted in significantly less thrombocytopenia and fibrinogen consumption as compared with injection of collagen alone. The results of both the in vitro and in vivo studies are consistent with the proposed antithrombotic effect of plasma fibronectin.

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PLASMA FIBRONECTIN (Fn), a high-molecular-weight glycoprotein, has been implicated in a wide variety of biological activities.[1-3] Among other things, plasma fibronectin has been demonstrated to bind to collagen,[4,5] fibrin,[6,7] and activated platelets.[8] Such observations have led to considerable speculation as to fibronectin's precise role in hemostasis and, in particular, platelet function. The situation is complicated by the presence of different isotypes of fibronectin (eg, the plasma form and the cell surface or tissue form). Platelets contain a form of fibronectin within their α-granules.[9,10] Studies by Plow et al[9] and Zucker et al[10] have demonstrated approximately 2 to 4 μg Fn/10^9 platelets in humans. On thrombin stimulation, the platelet fibronectin is secreted in concert with other granular proteins.[11,12] However, immunofluorescent microscopy indicates that there is essentially no fibronectin on the surface of "resting," unactivated platelets.[13] After thrombin stimulation, platelets express a specific, saturable receptor for plasma fibronectin.[8] Platelets spreading on collagen have also been reported to bind plasma fibronectin.[14] These findings suggest that fibronectin is involved in platelet function, particularly with regard to platelet–collagen interactions.

Bensusan et al[15] demonstrated that the addition of exogenous plasma fibronectin to collagen-induced platelet aggregation inhibited ^{14}C-serotonin release. This finding coincided with work in our laboratory suggesting that plasma fibronectin may exert an antithrombotic role in vivo.[16] Depletion of plasma fibronectin in rats by monospecific antibody led to a greater sensitivity to thrombotic stimuli and a significantly greater platelet consumption (thrombocytopenia) as compared with controls.[16] Several mechanisms might be involved in plasma fibronectin's proposed antithrombotic action with regard to platelets. One possibility is that fibronectin might directly inhibit platelet–collagen interactions.

The current study was designed to determine the effect of plasma fibronectin on collagen-induced platelet aggregation under defined in vitro and in vivo conditions. To facilitate comparison of in vitro and in vivo results, an homologous rat model was used.

MATERIALS AND METHODS

Male Sprague-Dawley rats (Taconic Farms, Germantown, NY) weighing 300 to 350 g were used for platelet and protein isolations. Animals were housed with veterinary supervision under controlled conditions, with food (Purina Rat Chow) and tap water ad libitum. All animals were anesthetized with diethyl ether or sodium phenobarbital before any surgical manipulation.

Adenosine diphosphate (ADP) and rat albumin were purchased from Sigma Chemical Co (St Louis). Purified α-thrombin was the generous gift of Dr John Fenton (NY State Health Laboratories, Albany). Stractan II was purchased in powder form from St Regis Co., Tacoma, Wash.) Buffers and solutions were prepared using distilled deionized water and sterilized by filtration using a 0.45-μm filter (Nalge Co, Rochester, NY).

Platelets were isolated from whole rat blood using the method of Corash et al.[17] Whole blood was drawn by venipuncture with an 18-gauge needle into a plastic syringe containing two parts 19% sodium citrate for every 100 parts blood. Platelet-rich plasma (PRP) was then prepared by adding 1 mL of a buffered saline glucose–citrate solution (BSG-citrate; 0.117 mol/L NaCl, 0.0136 mol/L sodium citrate, 0.011 mol/L glucose, 0.0086 mol/L Na_2HPO_4, and 0.0016 mol/L KH_2PO_4, pH 7.4) to 9 mL blood and centrifuging at 1,000 g for three minutes. This procedure was repeated three to five times until >95% of all platelets were obtained. The BSG-citrate–diluted PRP was layered over a discontinuous gradient consisting of 4 mL 20% Stractan II (isoosmotic arabinogalactan solution) and 3 mL 10% Stractan II in a 15-mL conical centrifuge tube. The tube was centrifuged for 15 minutes at 3,500 g, after which the plasma/BSG and 10% Stractan layers were aspirated. The platelet layer was then removed and washed free of Stractan by centrifugation for eight minutes at 1,200 g in excess BSG-citrate. The platelet pellet was resuspended in modified (calcium/magnesium-free) Tyrode's solution (0.137 mol/L NaCl, 0.0030 mol/L KCl, 0.012 mol/L $NaHCO_3$, 0.006 mol/L glucose, and 0.004 mol/L NaH_2PO_4, pH 7.4) or appropriate medium. This method of isolation removes all plasma proteins while causing minimal damage to the platelets. All procedures were carried out using plastic or siliconized glassware at room temperature unless otherwise specified. Platelet counts were performed using a hemocytometer (American Optical, Buffalo, NY)

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and Nikon phase contrast microscope (Nikon, Garden City, NY) after the method of Bjorkman.[18]

Rat platelet aggregation was measured in vitro according to the method of Born,[19] using a Payton Single Channel 300-B Aggregation Module (Payton Assoc, Inc, Buffalo, NY) and recorded with an Omniscribe B-5000 chart recorder (Houston Instruments, Austin, Tex). Aggregometry was performed at a block temperature of 37 °C and a stir bar speed of 900 rpm using a platelet concentration of 3×10^8 cells per milliliter. A typical aggregation assay consisted of 100 μL of isolated platelets in Ca^{++}/Mg^{++}-free modified Tyrode's solution, 10 μL $CaCl_2$ (0.001 mol/L final concentration), appropriate volumes of aggregating agents and plasma fibronectin in phosphate-buffered normal saline (PBS; 0.01 mol/L NaH_2PO_4/Na_2HPO_4, 0.15 mol/L NaCl, pH 7.4), or PBS vehicle alone. The assay mixture was brought up to 0.50 mL final volume using modified Tyrode's solution For most experiments, the platelet suspension in Tyrode's with calcium added was allowed to equilibrate with fibronectin or PBS while stirring for two minutes before the addition of collagen or other aggregating agent. When fibrillar (preformed) collagen was desired, the appropriate amount of acid-soluble collagen was added to the Tyrode's solution in an aggregation cuvette (with calcium and Tyrode's solution but without platelets and fibronectin) and allowed to form fibrils overnight at room temperature. Rat plasma fibrinogen (200 μg) was added to the cuvette for ADP-induced aggregations. To characterize the aggregation response, the following parameters were measured: the slope of the recorder tracing, which is an indicator of the rate of aggregation; the length of lag time from addition of the aggregating agent (collagen) to the onset of upward pen deflection; and the maximal scale deflection attained by the recorder tracing, which is an indicator of the completeness of the aggregation. Aggregation rate was measured by determining the rate of rise of the pen tracing for the first 30 seconds after leaving baseline (ie, after completion of the lag phase) and expressed as millimeters per minute. Lag time was expressed in minutes (chart speed equaled 25 mm/min). Maximum pen deflection was determined five minutes after the completion of the lag phase and expressed in millimeters. In almost all cases, aggregation was complete within four to five minutes and did not change when observed for a subsequent ten minutes.

The observations made with the Payton aggregometer were confirmed and extended using a Model 500 Whole Blood Aggregometer (Chrono-Log Corp, Havertown, Pa). Aggregation assays in this device were also performed at 37 °C with 3×10^8 platelets per milliliter and a stir bar speed of 900 rpm. A typical assay mixture contained 200 μL platelets, modified Tyrode's solution, 20 μL $CaCl_2$ (0.001 mol/L final concentration), 60 μL collagen (1 mg/mL stock concentration in 0.01 mol/L acetic acid), plasma fibronectin, and 40 μL Chrono-Lume Reagent (luciferase-luciferin reagent, Chrono-Log Corp) in a 1 mL final volume. An important feature of the Chrono-Log aggregometer is the ability to simultaneously measure changes in light transmittance and secretion of adenosine triphosphate (ATP), one of the constituents of the dense granule. Thus the kinetics of dense granule secretion could be followed continuously and compared with light transmittance data.

To determine the effect of fibronectin on in vivo collagen-induced platelet aggregation, male Sprague-Dawley rats weighing 200 to 300 g were anesthetized by intraperitoneal injection of 35 mg/kg sodium pentobarbital (Nembutal, Abbott, N Chicago). After the induction of surgical anesthesia, the tip of the tail was amputated to allow blood sampling without the use of indwelling cannulas or repeated venipuncture. This "tail sampling" method provides samples that have been shown previously to be reproducible.[20]

In vivo platelet aggregation was induced by injection of microfibrillar collagen (2 mg/kg body weight) suspended in PBS, pH 7.4, over approximately a one-minute time period. Injections were performed by way of the dorsal penis vein. Blood samples were taken just before injection (0 time) and at 5, 15, and 60 minutes after collagen injection. After the 60-minute sample, animals were sacrificed. Plasma fibronectin, fibrinogen, and fibrin(ogen) degradation products (FDPs) were also determined for each sample using immunochemical methods (see later). Because the first microcirculatory bed in which collagen could lodge would be the pulmonary circulation, lung wet-dry weight ratios were measured as an index of lung injury. For determination of wet-dry weight ratios, wet weights were measured, the lungs were then dried to constant weight, and the ratio of wet to dry weights calculated.

There were five treatment groups in the experiment, each group consisting of 12 animals. The injection protocol is shown in Table 1.

The key experimental groups are group 1, collagen injection followed by injection of phosphate-buffered saline (PBS) vehicle, and group 3, injection of collagen preincubated with plasma fibronectin (two times the amount of collagen on a per weight basis) for 30 minutes at room temperature followed by injection of PBS vehicle. Group 2 acted as a control for group 3, in that the same amount of fibronectin was injected as in group 3 but one minute *after* collagen injection (ie, the collagen was not "precoated"). This control necessitated a two-injection sequence, as shown in Table 1. Each injection was 2 mL in volume and given over approximately one minute. Groups 4 and 5 were the vehicle and the fibronectin alone control groups. Because the data were dependent in nature (ie, several measurements were made on each animal over time), the results were first analyzed by a repeated measures analysis of variance (BMDP.2V program), testing both treatment group effect and time effect.

Acid-soluble collagen (type 1) was isolated in a manner similar to that of Morin et al.[21] Rat tail tendon was excised and rinsed in cold saline, finely minced, and homogenized for five minutes in 0 5 mol/L acetic acid at 4 °C with a Brinkman homogenizer (Model PT10-35, Brinkman, Westbury, NY). All subsequent steps in the procedure were done at 4 °C. After homogenization, the suspension was stirred overnight and then centrifuged at 10,000 g for one hour to remove nonsolubilized material. Solid NaCl was added to the clarified homogenate until a 1 mol/L NaCl concentration was achieved. The precipitate formed was resuspended in 0.01 mol/L acetic acid and dialyzed extensively against this same solution. Collagen concentration was determined gravitometrically after lyophilization. Collagen purity was assessed by sodium dodecyl sulfate-polyacrylamide gradient gel (5% to 15%) electrophoresis under reducing conditions. For in vivo injection, fibrillar collagen was prepared by diluting purified acid-soluble collagen with PBS, pH 7.4, and incubating at 26 °C overnight. Before injection, fibrillar collagen was homogenized with a Brinkman Polytron.

Plasma fibronectin was isolated from rats by affinity chromatography on gelatin-Sepharose. The procedure is a modification[22] of the preparative gelatin-Sepharose column method used by Engvall and Ruoslahti.[4] Fibronectin concentration in plasma was measured by the electroimmunoassay or "rocket" immunoelectrophoresis method[23] using monospecific antirat fibronectin antibody prepared and isolated from rabbits as previously described.[24] Rat plasma fibrinogen was isolated by glycine precipitation,[25] and clottability was

Table 1. Injection Protocol

Treatment Group	First Injection	Second Injection
1	Collagen	Vehicle (PBS)
2	Collagen	Fibronectin
3	Collagen/Fibronectin	PBS
4	PBS	PBS
5	Fibronectin	PBS

measured by the procedure of Laki.[26] Fibrinogen concentrations were determined by radial immunodiffusion[27] also using monospecific antirat antibody raised in rabbits. FDPs were measured according to the method of Kaplan et al.[28] Concentrations of isolated proteins in solution were determined by the Lowry assay.[29]

Statistical analysis of data was performed with the assistance of the BMDP statistical software package[30] on an IBM 3033 computer. Comparison of two treatment groups was performed using a one-tailed Student's *t* test with the significance level set at $P < .05$. Comparison of more than two treatment groups was performed using a one-way (one-factor) analysis of variance (ANOVA). If the ANOVA showed a significant F ratio ($P < .05$), then either a Fisher's protected *t* test or Newman-Keuls multiple comparison test was applied to identify specific group differences. Lung wet-dry weight ratio data are not normally distributed. Therefore, statistical analysis was performed on the arc sin transformation of the raw data. In the in vivo platelet aggregation study, several measurements were made on the same animal over time. Data collected in this manner are dependent (ie, internally related). Therefore, these data were analyzed first with a Repeated Measures ANOVA to determine whether there was a significant difference ($P < .05$) due to treatment group as well as time effect. When this had been verified, each time period was analyzed by a one-way ANOVA, followed by multiple comparison tests. Data are expressed as the mean ± standard error of the mean (SE) unless otherwise stated.

RESULTS

The effect of plasma fibronectin on in vitro collagen-induced platelet aggregation was investigated using an isolated homologous rat system. Typical aggregation response curves using acid-soluble collagen and fibrillar collagen are depicted in Fig 1. The quantitative results are shown in Table 2. The effect of fibronectin on acid-soluble collagen-induced aggregation is shown in Fig 1A and Table 2. Increasing concentrations of plasma fibronectin significantly increased lag time (> threefold at 60 μg Fn as compared with PBS control) but did not affect aggregation maximum. The observations on in vitro aggregation made with the Payton aggregometer were confirmed and extended in a Chrono-Log Whole Blood Aggregometer using both luminescence and optical channels. The luminescence channel, however, enabled us continuously to measure the release of ATP as an indicator of dense granule release. Release of ATP is first detectable at the beginning of the shape change portion of the optical (transmittance) curve. This is consistent with the findings of others using human platelets.[31] In the presence of plasma fibronectin, ATP release occurs at the same relative position but is delayed in absolute time because of the increased lag time. The extent of ATP release (as compared with the standard) was the same for both control and fibronectin aggregations.

Table 2. Effect of Plasma Fibronectin on In Vitro Collagen-Induced Platelet Aggregation

Group	Lag Time (min)	Aggregation Rate (mm/min)	Maximum Aggregation (mm)
Acid-soluble collagen			
Control	2.10 ± 0.08	100.8 ± 6.2	143.0 ± 6.4
30 μg Fn	2.51 ± 0 07*	108.8 ± 4.6	141.2 ± 4.6
Control	0.81 ± 0.09	105.8 ± 3.6	140.2 ± 7.3
45 μg Fn	3.17 ± 0.14*	89.5 ± 17.0	147.0 ± 8.6
Control	0.98 ± 0.05	96.6 ± 7.6	138.0 ± 3.7
60 μg Fn	3.18 ± 0.08*	93.5 ± 2.1	134.6 ± 6.1
Fibrillar collagen			
Control	1.13 ± 0.13	102.7 ± 5.5	149.7 ± 4.1
30 μg Fn	1.68 ± 0.08*	71.3 ± 4.7*	144.7 ± 8.5
Control	0.81 ± 0 06	98.0 ± 1.2	154.0 ± 3 0
45 μg Fn	1.44 ± 0.13*	64.3 ± 4.1*	117.3 ± 5.6*
Control	0.58 ± 0.09	95.2 ± 5.2	162.2 ± 4.8
60 μg Fn	1.67 ± 0.08*	55.6 ± 8.1*	102.4 ± 8.1*

Platelet aggregation was initiated by the addition of 30 μg of acid-soluble type I rat collagen to a suspension of rat platelet (1.5 × 10^8) in Tyrode's solution with 1 mmol/L (final concentration) calcium added. Final cuvette volume was 0.5 mL. Plasma fibronectin in phosphate-buffered saline (PBS) in the amounts indicated was equilibrated with the platelets before collagen addition. Controls consisted of the appropriate volumes of PBS being added in lieu of fibronectin. Data are expressed as mean ± SE. Platelet aggregation also was initiated by the addition of 1.5 × 10^8 rat platelets in 100 μL Tyrode's solution to 30 μg of preformed fibrillar rat collagen (type I). The indicated amounts of fibronectin (or equal volume of PBS vehicle) were added just before platelet addition. The fibrillar collagen was formed by overnight incubation at 26 °C in 250 μL Tyrode's solution with calcium added Fn, fibronectin.

*Significantly ($P < 05$) different from control value (n = 5 to 7 in each group).

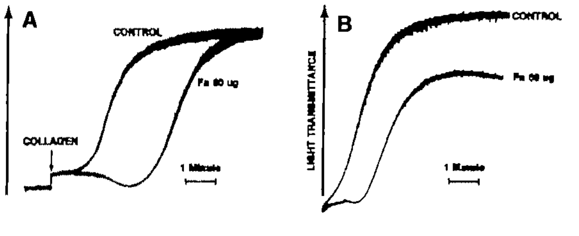

Fig 1. (A) Typical acid-soluble collagen-induced rat platelet aggregation curves. Type I collagen (30 μg) was added to platelets (1.5 × 10^8 cells) suspended in Tyrode's solution with either fibronectin (Fn) in PBS or PBS vehicle alone (control). The final cuvette volume was 0.5 mL. (B) Typical fibrillar collagen-induced rat platelet aggregation curves. Aggregation was initiated by the addition of 100 μL of 1.5 × 10^8 platelets in Tyrode's solution to fibrillar collagen that had been preformed in the cuvette. Plasma fibronectin or PBS control was added just before platelet addition. The final cuvette volume was 0.5 mL.

The lag time phase of the platelet aggregation curve depends on at least two processes when acid-soluble ("monomeric") collagen is used to induce aggregation. One process is the platelet-collagen interaction, which was of prime interest to this study. Because monomeric collagen will not activate platelets, the collagen must first undergo fibril formation before induction of aggregation. Collagen fibrillogenesis complicates the interpretation of the experiments above, since fibronectin has been reported to inhibit in vitro collagen fibrillogenesis.[32] To eliminate this variable, collagen fibrils were preformed by overnight incubation and then platelets were added with fibronectin or PBS vehicle control. Typical response curves for fibrillar collagen are shown in Fig 1B and, quantitatively, in Table 2. At all the concentrations studied, fibronectin significantly increased lag time and decreased aggregation rate. At 45 and 60 μg Fn, the aggregation maximum was also significantly inhibited. Because collagen fibrillogenesis was not occurring, these data are consistent with plasma fibronectin having an inhibitory effect on the initial platelet–collagen interaction.

The effect of fibronectin on two other aggregating agents, ADP and purified thrombin, was also studied. Even using 200 μg Fn/mL, a concentration almost double those that inhibited in collagen-induced aggregation, fibronectin had no effect on the rate or extent of either ADP or thrombin-induced platelet aggregation (Table 3). Thus the effect of fibronectin, at the concentrations used, appears to be specific to the collagen–platelet interaction and not an inhibition of the platelet–platelet interaction, which has been reported to occur with much higher concentrations of fibronectin.[33]

As a model of collagen-induced platelet aggregation in vivo, anesthetized rats were injected IV with collagen (2 mg/kg body weight) suspended in PBS. Five treatment groups were established with a two-injection protocol, as shown in Table 1. The basic hypothesis was that if plasma fibronectin did inhibit collagen-induced platelet aggregation, then collagen preincubated ("precoated") with fibronectin would cause less thrombocytopenia or platelet consumption than injection of collagen alone.

The changes in circulating platelet number after in vivo collagen injection are shown in Table 4. Injection of collagen alone in vehicle (group 1) caused a 29% drop in platelet count within five minutes. Over the next hour, however, platelet count returned to preinjection levels. Injection of collagen preincubated with two times its weight of plasma fibronectin (group 3) resulted in only a 17% decrease in platelet count at the five-minute time point and was significantly ($P < .05$) different from collagen alone (group 1). Group 2 (collagen followed by fibronectin injection) was not statistically different from group 1 at five minutes. In other words, at the first measurement time after injection, collagen preincubation with fibronectin (group 3) appeared to protect against the thrombocytopenia after injection of collagen alone (group 1), whereas injection of collagen followed by injection of fibronectin (group 2) was less protective. Injection of vehicle alone (PBS) resulted in a decreased platelet count due to "dilution effects." The injection of fibronectin alone (group 5), which served as one of the controls, resulted in a rise in platelet counts by five minutes postinjection. At 15 and 60 minutes postinjection, only the fibronectin control group (group 5) was significantly different from the collagen group (group 1).

Table 3. Effect of Plasma Fibronectin on Platelet Aggregation Induced by ADP and Thrombin

	Fibronectin (μg)	Aggregation Rate (mm/min)	Maximum Aggregation (mm)
ADP	0	114.3 ± 6.7	147.0 ± 4.0
	100	114.4 ± 6.6	143.7 ± 8.2
Thrombin	0	131.3 ± 3.9	156.8 ± 4.2
	100	141.2 ± 4.9	168.3 ± 3.6

Platelet aggregation was initiated by the addition of 10 μL of either ADP (2μmol/L final concentration) or purified human thrombin (1 unit) to 1.5×10^8 rat platelets suspended in modified Tyrode's solution with calcium (1 mmol/L final concentration). For ADP-induced aggregation, 200 μg rat fibrinogen was added before the ADP. All other conditions were the same as those used in collagen-induced aggregation. Data are expressed as mean ± SE ($n = 5$). No significant differences were detected.

Measurement of fibronectin during the experiment (Table 5) revealed that the only significant changes that occurred were elevations in fibronectin concentration in groups 2 and 5. Group 2 (collagen followed by fibronectin) was significantly different from groups 1, 3, and 4 at five minutes postinjection, as was group 5 (injection of fibronectin alone). At this time point, groups 2 and 5 were not significantly different from each other. By 15 minutes postinjection, fibronectin levels in group 2 had returned to baseline values, while those of group 5 remained significantly elevated. The fact that there was no significant elevation in plasma fibronectin levels in group 3 (collagen preincubated with fibronectin) animals indicates that most of the injected fibronectin was associated with the injected collagen.

Measurement of plasma fibrinogen levels indicated that injection of collagen (group 1) resulted in a rapid consumption or depletion of fibrinogen. These data are presented in Table 6. Within five minutes, fibrinogen levels dropped 36% in the collagen group (group 1) and stayed depressed throughout the 60 minutes of the experiment. In contrast, collagen preincubated with fibronectin (group 3) caused only a 14% drop in fibrinogen concentration at the five-minute time point, which was significantly ($P < .05$) different from group 1. After a second drop at 15 minutes postinjection, fibrinogen levels in group 3 returned to baseline at 60 minutes. Injection of collagen followed by fibronectin (group 2) was not significantly different from injection of collagen alone (group 1) at any time. Thus, much like the observations made on platelet counts, collagen preincubated with fibronectin (group 3) appeared to protect against fibrinogen consumption when compared with injection of collagen alone (group 1), whereas collagen followed by fibronectin afforded no protection.

Although measurements of FDPs were made, the amount of FDPs generated was below the detection limits of the assay. In our hands, this assay for FDPs has shown significant changes in FDP levels in other rat models of thrombosis, such as intraperitoneal thrombin injection.[16,21] Therefore, it appeared that during the one hour after collagen injection, there was little or no detectable fibrinolysis.

Lung wet-dry weight ratios were measured as an index of lung injury as induced by the collagen injection (Table 7). Injection of collagen alone (group 1) caused a significant ($P < .05$) increase in lung wet-dry weight ratios when compared with injection of vehicle (group 4). Injection of collagen preincubated with fibronectin (group 3) and injection of collagen followed by injection of fibronectin (group 2) resulted in lung wet-dry weight ratios that were significantly lower than those of group 1. Groups 2, 3, and 5 were not statistically different from vehicle injection (group 4). The vehicle group had a wet-dry ratio of 4.89 ± 0.15.

DISCUSSION

Plasma fibronectin significantly ($P < .05$) inhibits collagen-induced platelet aggregation in the isolated rat platelet system. Using homologous cells and proteins, fibronectin increased the lag time using acid-soluble collagen to induce

Table 4. Changes in Circulating Blood Platelet Number During In Vivo Collagen-Induced Platelet Aggregation

	Treatment Group				
Time (min)	1 Collagen	2 Collagen Followed by Fn	3 Collagen Preincubated With Fn	4 PBS Vehicle	5 Fibronectin
0	596.9* ± 31.1	536.7 ± 35.5	653.5 ± 50.8	586.2 ± 18.5	670.0 ± 40.1
5	423.8 ± 23.5	433.9 ± 34.6	537.3† ± 42.7	517.3† ± 28.4	695.2† ± 47.1
15	504.8 ± 33.8	462.1 ± 41.0	591.8 ± 58.2	557.3 ± 15.5	613.1† ± 29.5
60	559.2 ± 16.3	534.4 ± 40.2	680.2 ± 46.4	589.8 ± 31.7	735.2† ± 52.7

Circulating blood platelets were measured at the times shown by hemocytometer. Platelet counts were performed after IV injection of 2 mg/kg collagen (group 1), collagen followed one minute later by injection of 4 mg/kg fibronectin (group 2), collagen preincubated with 4 mg/kg fibronectin (group 3), PBS vehicle control (group 4), or 4 mg/kg fibronectin control (group 5) into anesthetized rats. Data are expressed as the mean ± SE and each group consisted of 12 animals. Fn, fibronectin.

*Platelet count ($\times 10^3/\mu L$)

†Significantly ($P < .05$) different from collagen group (group 1). See Materials and Methods for details of statistical analysis.

Table 5. Changes in Plasma Fibronectin Concentration (μg/mL) During In Vivo Collagen-Induced Platelet Aggregation

	Treatment Group				
Time (min)	1 Collagen	2 Collagen Followed by Fn	3 Collagen Preincubated With Fn	4 PBS Vehicle	5 Fibronectin
0	344.7 ± 22.4	376.5 ± 40.4	402.4 ± 26.9	382.6 ± 29.8	369.4 ± 12.9
5	311.3 ± 21.2	430.4* ± 34.6	384.1 ± 32.6	362.9 ± 27.8	398.6* ± 10.9
15	333.1 ± 28.2	373.7 ± 34.7	386.5 ± 31.3	351.2 ± 31.6	398.6* ± 18.8
60	319.6 ± 33.1	397.7 ± 34.2	361.1 ± 32.9	338.9 ± 34.6	399.3* ± 9.4

Plasma fibronectin levels were measured by rocket electroimmunoassay as previously described,[24] using monospecific antirat antibody raised in rabbits. Data are examined as mean ± SE. Each treatment group consisted of 12 animals Fn, fibronectin

*Significantly ($P < .05$) different from collagen group (group 1) at that time.

Table 6. Changes in Fibrinogen Concentration (mg/mL) During In Vivo Collagen-Induced Platelet Aggregation

	Treatment Group				
Time (min)	1 Collagen	2 Collagen Followed by Fn	3 Collagen Preincubated With Fn	4 PBS Vehicle	5 Fibronectin
0	2.32 ± 0.46	2.88 ± 0.38	2.44 ± 0.38	3.21 ± 0.35	2.69 ± 0.39
5	1.49 ± 0.09	2.12 ± 0.37	2.09* ± 0.24	2.47* ± 0.36	2.45* ± 0.36
15	1.48 ± 0.23	1.99 ± 0.37	1.75 ± 0.16	2.70 ± 0.46	2.61* ± 0.41
60	1.53 ± 0.30	2.06 ± 0.33	2.58* ± 0.35	1.82 ± 0.42	2.88* ± 0.53

Fibrinogen concentrations were measured by radial immunodiffusion as previously described,[28] using monospecific antirat antibody raised in rabbits. Data are expressed as mean ± SE. Each treatment group consisted of 12 animals Fn, fibronectin.

*Significantly ($P < .05$) different from collagen group (group 1)

Table 7. Lung Wet-Dry Weight Ratios

Treatment Group	1 Collagen	2 Collagen Followed by Fn	3 Collagen Preincubated With Fn	4 PBS Vehicle	5 Fibronectin
	5.64 ± 0.15	5.24 ± 0.11	5.22 ± 0.10	4.89 ± 0.15	4.89 ± 0.10

After the 60-minute blood sample, animals were sacrificed and the lungs rapidly excised, rinsed in cold saline, and weighed. Dry weight was measured after oven drying to a constant weight. Data are expressed as mean ± SE. Each group consisted of 12 animals. Values for groups 2 through 5 were significantly ($P < .05$) different from the collagen group (group 1) as determined by one-way analysis of variance and a Newman-Keuls multiple comparison test performed on an arc sin transformation of the raw ratio data. Fn, fibronectin.

aggregation. In part, this may be attributable to an inhibition of collagen fibrillogenesis as reported by Kleinman et al.[32] However, when fibrillar collagen was used to induce aggregation, plasma fibronectin still increased lag time. Furthermore, use of higher concentrations of fibronectin (1.5 × and 2.0 × collagen, wt/wt) resulted in a significant decrease in aggregation rate and the maximum extent of aggregation, as if fibronectin had blocked platelet binding sites on collagen, limiting the stimulus for aggregation.

The inhibitory effect of plasma fibronectin, at the concentrations used, appeared to be specific for collagen in that neither ADP- nor thrombin-induced aggregation was affected even in the presence of 200 μg Fn/mL. This concentration was almost twice that at which inhibition of collagen-induced aggregation could be detected. Santoro[33] has reported that plasma fibronectin can inhibit in vitro human platelet aggregation as induced by thrombin or ionophore A23187. In that system, the threshold for fibronectin's inhibitory effect was above levels used in this study, with maximal inhibition occurring at 500 μg Fn/mL. These concentrations are significantly greater than those needed to inhibit collagen-induced aggregation in our homologous rat system. Thus, while at first glance our observations might appear contrary to those of Santoro, this is not the case at all. We have demonstrated a specific inhibitory effect of fibronectin on collagen-induced aggregations at concentrations lower than those Santoro needed to show any inhibitory effect of fibronectin on thrombin- or A23187-induced aggregation. These observations suggest that fibronectin can inhibit platelet aggregation through two mechanisms. Low concentrations of fibronectin may inhibit the platelet–collagen interaction, whereas high concentrations may directly influence the platelet–platelet interactions.

Results of the in vivo collagen-induced platelet aggregation experiment were consistent with fibronectin's inhibitory effect in the in vitro experiments. Preincubation ("precoating") of collagen with fibronectin (group 3) before IV injection into anesthetized rats resulted in significant protection against the thrombocytopenia, fibrinogen consumption, and increase in lung wet-dry weights when compared with injection of fibrillar collagen alone (group 1). Injection of the same amount of fibronectin one minute after the collagen (group 2) was not as effective in preventing the thrombocytopenia and fibrinogen consumption. Injection of fibronectin alone (group 5) caused a significant rise in circulating platelet count, presumably by triggering release of a temporarily sequestered platelet pool. The argument might be raised that the protective effect of platelet consumption seen with group 3 (collagen precoated with fibronectin) was simply release of a new pool of platelets due to increased circulating fibronectin. However, plasma fibronectin was not elevated in group 3. Group 2, which did not show a significant increase in plasma fibronectin at five minutes, was not as effective in preventing the thrombocytopenia as treatment group 3. Furthermore, injection of fibronectin one minute after collagen injection (group 2) did not prevent fibrinogen consumption at any time. Therefore, the protective effect seen with treatment group 3 is not attributable to elevated fibronectin levels in those animals. Rather, the data suggest that for fibronectin to exert its protective (antithrombotic) effect, it must be intimately associated with the collagen.

The influence of plasma fibronectin on the collagen-induced changes in lung wet-dry weight ratio appears more complex. Here both group 2 and group 3 treatments had significantly lower ratios than that of group 1 (collagen alone). Because an increase in wet-dry weight ratio could reflect both thrombosis and edema formation, different fibronectin effects may come into play with group 2 *v* group 3. Whereas the collagen precoated with fibronectin group (group 3) may have resulted in less pulmonary platelet aggregation and thrombosis, the elevated plasma fibronectin levels of group 2 may have been acting to limit pulmonary edema formation by influencing vascular permeability.[34] Obviously, caution must be used in interpreting the results of the in vivo collagen injection study because of the complexities of the whole animal model. Nevertheless, the results of the in vivo collagen-induced platelet aggregation study are consistent with the more defined in vitro platelet aggregometry. This suggests that plasma fibronectin may indeed play a physiologically significant antithrombotic role by inhibiting or limiting platelet–collagen interactions.

The mechanism of plasma fibronectin's inhibitory effect on collagen-induced platelet aggregation is not yet clear. It is apparent that inhibition occurs in the early phases of platelet aggregation (ie, the lag time). The timing of this inhibition suggests that fibronectin may be interfering with platelet–collagen binding (adhesion) or perhaps platelet activation. Santoro[33] reported inhibition of thrombin-induced aggregation with 500 μg Fn/mL but found no difference in serotonin release in the presence of fibronectin. Therefore, he concludes that under his conditions, fibronectin inhibited some process after primary platelet activation. However, the possibility still exists that fibronectin does inhibit platelet activation induced by collagen. This question is currently under study.

Another possible mechanism for fibronectin's inhibitory action on platelet aggregation is that fibronectin might decrease the platelet adhesion response to collagen. Sochynsky et al[35] reported that preincubation of fibrillar collagen inhibited human platelet adhesion. Most other studies, however, have reported that fibronectin enhances platelet adhesion or attachment and spreading to collagen.[36,37] Our own experiments on the effect of fibronectin on platelet adhesion to collagen in a homologous rat model (Moon and Kaplan, unpublished observations, 1983) have yielded results virtually the same as those reported by Koteliansky et al[37] for human platelets. A major limitation of most platelet adhesion/attachment studies is that they were done under nonflow, unstirred conditions. As has been well demonstrated, changes in shear stress and shear rate can be important parameters in the function of proteins involved in platelet attachment to collagen.[38] A recent report by Houdijk et al[39] describes the influence of fibronectin and von Willebrand factor (vWF) on the interaction of platelet with acid-soluble and fibrillar collagens types 1 and 3 under defined flow conditons. Optimal platelet adhesion to acid-soluble (monomeric) collagen was dependent on both vWF and fibronectin. vWF was required only at relatively high shear rates, whereas platelet deposition on acid-soluble collagen was depressed at all shear rates when fibronectin-free plasma was used as a perfusate. Similar results were obtained with fibrillar collagen. However, when preincubation of collagen with vWF and fibronectin was performed, fibrillar collagen showed a requirement for only vWF and not fibronectin. These results are in contrast to preincubation studies with acid-soluble collagen, which demonstrated a requirement for both vWF and fibronectin. These experiments point out the differences that can arise in using acid-soluble *v* fibrillar collagen. While these reports suggest a role for fibronectin in mediating platelet attachment to and spreading on collagen, they do not explain the mechanism of fibronectin's inhibitory action.

The study reported here was designed to address the potential antithrombotic role of plasma fibronectin in collagen-induced platelet aggregation. The influence of neither the cell surface (tissue) fibronectin nor the platelet's own fibronectin has not been delineated. Houdijk and Sixma[40] suggest that fibronectin in the subendothelium is important for platelet adhesion. Using human umbilical artery subendothelium in an annular perfusion chamber, they found that preincubation of the vessel with antifibronectin IgG reduced indium-111 platelet deposition. This effect was significant at a shear rate of 800 S^{-1} or greater, and we suggest that tissue fibronectin enhances platelet attachment. However, despite the apparent similarities of the in vitro actions of the fibronectins, they may play very different roles physiologically in the platelet–collagen interaction.

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Human Recombinant Interferon α-2C Enhances the Expression of Class II HLA Antigens on Hairy Cells

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Isolated splenic hairy cells from three untreated patients were cultured in presence of recombinant human interferon alpha-2C (IFNα). Ultrastructural cytochemistry and immunophenotype analysis with a large panel of monoclonal antibodies were performed to study cellular modification induced by IFNα. Hairy cells showed a typical phenotype: SmIg$^+$, B1$^+$, BA1$^\pm$, anti-Tac$^+$, OKDR$^+$, Leu-M5$^+$, HC2$^+$, TRAP$^+$, myeloperoxidase$^-$. Under our experimental conditions, we found no direct cytotoxic effects or significant variations in morphology, cytochemistry, and percentage of reactivity with the tested monoclonal antibodies. After culturing in the presence of different doses of IFNα, we observed a significant enhancement of the expression of class II HLA antigens as demonstrated by increased fluorescence for OKDR, OKIa, Leu-10 at fluorescence-activated cell sorting analysis. In agreement with this finding IFNα-treated hairy cells showed an increased stimulatory capacity *v* allogeneic T cells in one-way mixed lymphocyte reaction. To our knowledge this is the first report describing the induction of class II HLA antigens on hairy cells by IFNα.

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THE MECHANISMS OF antitumor action of the interferons (IFNs) have not yet been clearly established. They are postulated to have a direct effect on tumor cell growth and to enhance the host's immune responses, properties that could be of great importance in inhibiting tumor proliferation.[1-3] Recently, moreover, it has been demonstrated that IFN may act by promoting cell differentiation in tumor cell systems.[4,5] Next to the morphological and immunophenotypic differentiating effect, their most striking characteristic is an ability to enhance in vitro the expression of class I and II HLA antigens.[6-8] In particular, IFNγ has been shown to induce Ia antigens on B lymphomas and macrophagic cell lines.[9] Considering their natural origin, it would be interesting if IFNs had a "physiologic" antitumor effect due to their differentiating and modulating properties.

Hairy cell leukemia (HCL) is a B cell tumor[10] with a slow proliferative rate in which IFNα has recently been shown to have a clear antitumor effect.[11] With the aim of clarifying the action of IFN in this malignancy, we studied isolated splenic hairy cells in liquid phase cultures in presence of recombinant human IFN type alpha-2C.

MATERIALS AND METHODS

Patients. Three untreated patients with HCL were studied. Diagnosis was based on the clinical picture, light microscopy, and transmission electron microscopy (TEM) of bone marrow biopsies or isolated peripheral blood mononuclear cells, and the presence of tartrate-resistant acid phosphatase activity. Each patient underwent splenectomy because of enlarging spleen and/or severe cytopenia.

Isolation of hairy cells Splenic specimens were teased through a stainless steel mesh sieve to obtain a single cell suspension, which was centrifuged on a Ficoll-Hypaque gradient (MSL, Eurobio, Paris) The mononuclear cells recovered included more than 80% hairy cells at ultrastructural analysis Except for the ultrastructural studies, all marker tests and function assays were performed more than once on fresh and cryopreserved cells.

Cell cultures. Cells were cultured at a concentration of 1.5 × 10^6 in RPMI 1640 medium (Flow Laboratories, Irvine, UK) supplemented with 20% heat-inactivated fetal calf serum (FCS, GIBCO, Grand Island, NY) and antibiotics with or without IFNα (recombinant human interferon type alpha-2C, Boehringer, Ingelheim, FRG) at concentrations of 20, 100, and 500 IU/mL. Cultures were done in plastic Petri dishes (Sterilin Ltd, Teddington, UK) and incubated for 48 or 96 hours at 37 °C in a 5% CO_2 humidified incubator. In case 2 only, an additional set of cultures was carried out in which 20 IU/mL IFNα was added every 24 hours for four days of culture. Viability tests with the trypan blue exclusion method and all of the following tests were performed at the beginning and end of cultures.

Immunophenotype determination. We studied surface membrane immunoglobulins (SmIg) by direct immunofluorescence using fluorescein isothiocyanate-conjugated (FITC) polyclonal rabbit antibodies to human heavy and light chains (Dakopatts, Glostrup, Denmark) as previously described.[12]

Cell staining by monoclonal antibodies (mAb, listed in Table 1, references 13–16) was done by direct or indirect immunofluorescence methods using as second-step reaction FITC-labeled rabbit antimouse $F(ab)'_2$ fragments (Nordic, Immunological Laboratories, The Netherlands).

Before staining, the cells were incubated at 37 °C for ten minutes with acetate buffer, then for two hours in medium, and, subsequently, with rabbit nonimmune serum to eliminate cytophilic Ig and nonspecific Fc fragment binding. Samples were analyzed with a Leitz Dialux 20EB fluorescence microscope (Leitz, Wetzlar, Germany); fluorescence intensity of the cells was determined by flow cytometry on FACS 420 (Becton Dickinson, Mountain View, Calif) and on Spectrum III (Ortho Diagnostic Systems, Westwood, Mass). Cells (10,000) were scored, and the number of positive cells was calculated after subtracting the reactivity of cells stained with FITC rabbit antimouse antiserum alone.

Mouse rosettes. Mouse (M) rosettes were tested as previously described [12]

Cytoplasmic Ig. Cytoplasmic immunoglobulins (CyIg) were evaluated with the method described by Vogler et al[17] using FITC polyclonal rabbit antibody to heavy and light chains in appropriate dilutions (Dakopatts, Glostrup, Denmark). A Leitz Dialux 20EB fluorescence microscope was used to observe the slides.

Ultrastructural study. In addition to conventional analysis, splenic hairy cells were examined with TEM. Routine morphology was carried out on the whole series of samples as described elsewhere.[18] The Graham and Karnovsky technique was used to detect the presence of myeloperoxidase reaction[19] in hairy cells before and after culture.

The immunogold staining reaction[20] was also performed on some

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Table 1. Antibodies and Their Specificity

Antibody	Specificity	Source
OKIa1	Human Ia antigens, B lymphocytes, monocytes, activated T lymphocytes	Ortho Pharmaceutical Corp, Raritan, NJ
OKDR	HLA-DR monomorphic antigens on B lymphocytes, monocytes, activated T lymphocytes	Ortho Pharmaceutical Corp, Raritan, NJ
OKM1	Monocytes, granulocytes, natural killer cells	Ortho Pharmaceutical Corp, Raritan, NJ
BA1	B lymphocytes, non-T/non-B ALL, pre-B ALL, CLL, malignant lymphoma, granulocytes	Hybritech Inc, San Diego
B1	B lymphocytes	Coulter Immunology, Hialeah, Fla
Leu-1	Peripheral T cells, thymocytes, B CLL	Becton Dickinson, Sunnyvale, Calif
Leu-2a	T suppressor/cytotoxic cells	Becton Dickinson, Sunnyvale, Calif
Leu-3a	T helper/inducer cells	Becton Dickinson, Sunnyvale, Calif
Leu-7	NK and K cells	Becton Dickinson, Sunnyvale, Calif
Leu-10	HLA-DC/DS antigens on B lymphocytes, B cell lines	Becton Dickinson, Sunnyvale, Calif
Leu-11a	NK and K cells, neutrophils	Becton Dickinson, Sunnyvale, Calif
FMC32	Monocytes, macrophages[13]	Gift of H. Zola, Bedford Park, Australia
αHC2	Hairy cells, leukemic myeloblasts, some lymphomas[14]	Gift of D. Posnett, Rockefeller University, New York
Leu-M5	Hairy cells, monocytes[15]	Gift of H. Stein, Freie Universität, Berlin
Anti-Tac	Membrane receptor for interleukin 2 on malignant and activated T cells[16]	Gift of T.A. Waldmann, National Cancer Institute, Bethesda, MD

samples selected for immunoultrastructural investigations because previous immunofluorescence screening had presented problems of interpretation; nonhomogeneous expression of a surface antigen with a homogeneous cell population, unexpected positivity for an mAb usually nonreactive with hairy cells, and a large increase or decrease of expression of an mAb were the most frequent reasons why immunogold staining at ultrastructural level was performed. Ultrathin sections were examined before and after staining with a Philips 410 TEM.

Mixed lymphocyte reaction (MLR). T lymphocytes from four healthy donors (T_1 through T_4) were purified as previously described[21] and used as responder cells. Purified spleen hairy cells from cases 2 and 3, cultured for 48 hours with or without 100 IU IFNα, as described previously, and subsequently irradiated with 3,000 rad, were used as stimulator cells (T_1 and T_2 cells *v* hairy cells from case 2, T_3 and T_4 cells *v* hairy cells from case 3). Cocultures were established in quintuplicate, incubating 10^5 responder cells with 2.5, 5.0, and 10.0 × 10^4 stimulator cells in RMPI 1640 medium supplemented with 20% heat-inactivated FCS and antibiotics in round-bottomed wells of Microtest plates (Sterilin) at 37 °C in a 5% CO_2 humidified incubator for seven days. To evaluate cell proliferation, 1 μCi ^{3}H-thymidine (2 Ci/mmol, Radiochemical Centre, Amersham, UK) was added to the cultures 18 hours before harvesting. ^{3}H-Thymidine uptake was determined in a Packard tri-carb liquid scintillation counter. Conventional mixed lymphocyte cultures (MLC) were carried out in the same way, culturing 10^5 purified peripheral T_1 through T_4 cells with 10^5 allogeneic peripheral non-T cells treated with 5,000 rad.[22] Controls consisted of responder and stimulator cells cultured alone.

Statistical analysis. The statistical significance of data was evaluated with Student's *t* test.

RESULTS

Changes in morphology, cytochemistry, and immunophenotype in splenic hairy cells cultured with IFNα. After separation, the recovered cell fraction consisted mainly of mononuclear cells in which typical ultrastructural features of hairy cells could be found. At TEM, more than 80% of the cells had oval and deeply indented nuclei, numerous cytoplasmic vesicles, mitochondria, and characteristic narrow-based long projections at the periphery (Fig 1A). Graham and Karnovsky's myeloperoxidase reaction was negative.[19] The cell surface antigen expression detected by mAb in fresh spleen cells from our patients is reported in Table 2. All three cases displayed a B cell phenotype; a high percentage of cells expressed monoclonal-type SmIg, B1 antigen, and class II HLA antigens. Intensity of staining with Leu-10 was weaker than that with OKDR and OKIa1 but present on most cells. Positivity with Leu-10 in hairy cells was also less intense than that usually observed in normal circulating B lymphocytes. Leu-M5 staining was strongly positive in most hairy cells in contrast to BA1 antigen expression, which varied in the three cases. Positivity to anti-HC2 and anti-Tac was weak but detected in a high percentage of cells in all the cases, whereas OKM1 positivity was observed only in case 3.

No significant variations in viability, cell number, morphology at light microscopy, and cytochemcial pattern were observed at any culture time point, irrespective of the presence or amount of IFNα. No CyIg or M rosettes were detected either before or after culture.

After culture with or without IFNα, ultrastructural morphological changes appeared in the cells, indicating increased intracellular protein synthesis. More than 50% of the cell population had a large Golgi apparatus, numerous short strands of endoplasmic reticulum, and a very high number of cytoplasmic vesicles and pale granules. The Graham and Karnovsky myeloperoxidase reaction continued to be negative (Fig 1B through D).

We did not find any significant variation in percentage positivity for the various surface antigens investigated except in case 2. In all cultures of cells from this patient, we observed increased positivity to OKM1, which went from a low basal level (6%) to 50% and 60%, respectively, for cells exposed and unexposed to IFNα. On the other hand, at ultrastructural analysis, we observed persistent myeloperoxidase negativity as well as low expression (<5%) of other mAbs with known macrophage–monocyte specificity, such as Leu-M1 and FMC32 (Fig 1C).

The only IFN-induced variation we observed was in intensity of expression of class II HLA antigens. In all three cases studied, incubation of spleen hairy cells with IFNα clearly enhanced the intensity of surface expression antigens detected by OKDR, OKIa1, and Leu-10. This effect was

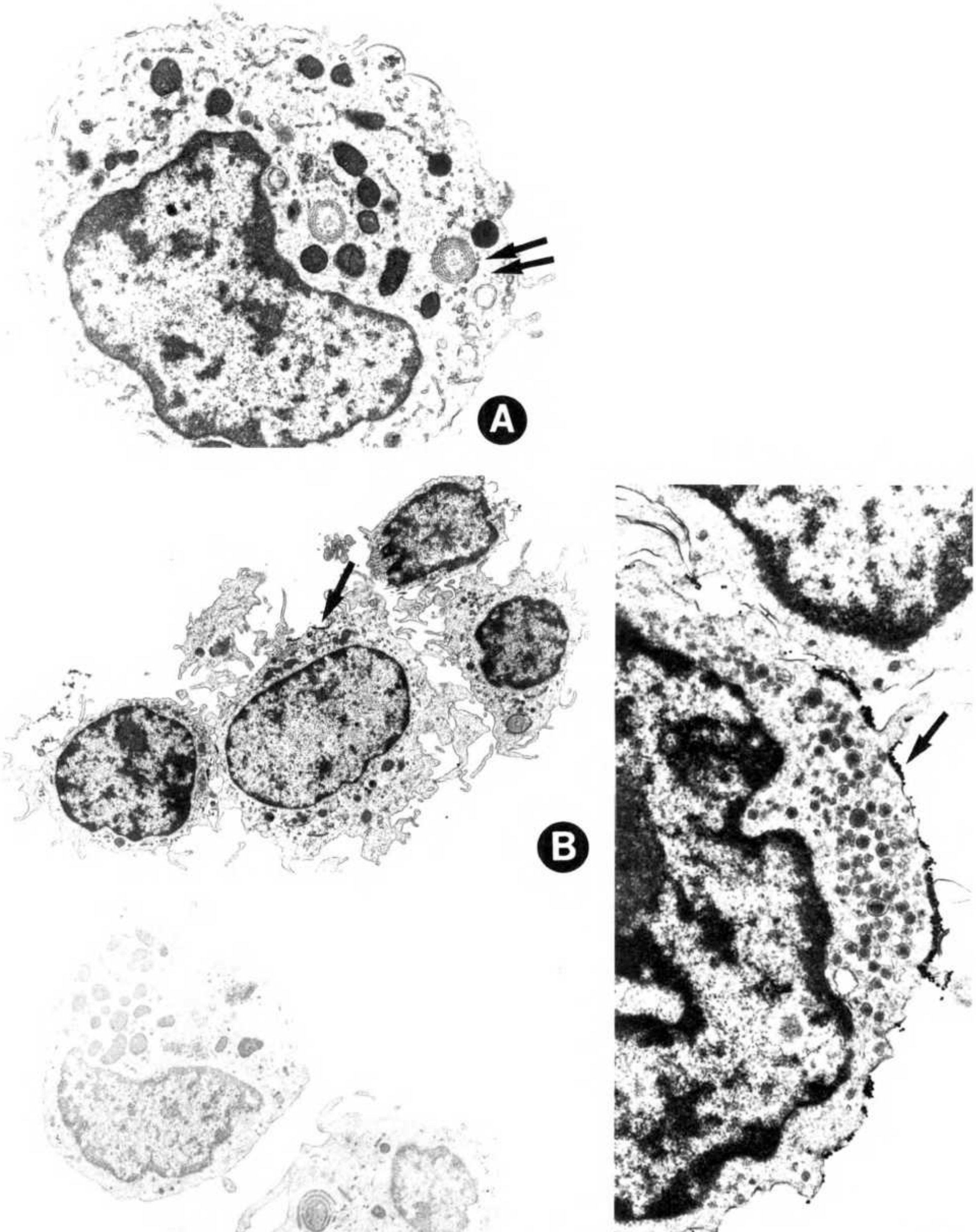

Fig 1. Splenic hairy cell suspension. (A) Oval and indented nuclei, cytoplasmic vesicles, and ribosome–lamella complexes are typical ultrastructural features of hairy cells (PbUa; original magnification ×10,000; current magnification ×7,700). (B) After culture in presence of IFN. The cells are positive for Leu-M5 (single arrow) at immunogold staining (PbUa; original magnification ×10,000; current magnification × 8,100). (C) After culture in presence of IFN. The cells are myeloperoxidase negative with Graham and Karnovsky's method (unstained; original magnification ×7,000; current magnification ×5,390). (D) Detail of cultured hairy cells strongly positive for Leu-M5 (single arrow). The increased number of cytoplasmic vesicles suggests active cell synthesis (PbUa; original magnification ×26,000; current magnification ×20,800).

Table 2. Phenotype of Fresh Mononuclear Cells From Spleen of Patients With HCL

	Positive Cells (%)		
	Patient 1	Patient 2	Patient 3
SmIg	60δλ	80γκ	70μδλ
OKIa1	83	75	80
OKDR	82	84	82
Leu-10	81	78	75
B1	75	85	84
BA1	5	25	50
Anti-Tac	70	60	80
Leu-M5	83	74	85
αHC2	76	84	78
OKM1	10	6	75
Leu-M1	5	3	5
FMC32	6	1	3
Leu-1, -2, -3, -7, -11	<5	<5	<5

shown by fluorescence microscopy, and even more clearly by cytofluorimetric analysis, and was detectable already at 48 hours' culture and with IFN concentrations as low as 20 IU/mL. Possibly due to some degree of activation induced by FCS, intensity of expression of these surface antigens was slightly increased in hairy cells cultured in the absence of IFN compared with freshly stained cells. However, this enhancement was significantly lower than that observed in the presence of IFN. FACS histograms derived from case 2 hairy cell analysis (Fig 2) show the characteristics of enhancement of class II HLA antigen expression, which were almost identical to those of cases 1 and 3 (not shown). The addition of a neutralizing concentration (1 μg/10 IU IFN) of an anti-IFNα mAb (EBI1, kindly provided by Dr G.R. Adolf, Ernst Boehringer Institut, Vienna) at the start of cultures of case 2 hairy cells with 100 IU/mL IFN was found to inhibit the enhancement of class II HLA antigen expression significantly compared with hairy cells cultured with IFNα and without EBI1. This enhancement was not significantly modified when hairy cells from case 2 were preincubated with anti-Tac for one hour at 4 °C.

Stimulatory capacity in MLR of spleen hairy cells cultured with IFNα. The increase in class II HLA antigens expression after IFN treatment in vitro was accompanied by enhancement of the stimulatory activity of spleen hairy cells against allogeneic purified peripheral T lymphocytes in one-way MLC. The responsiveness of these T cells was previously shown to be in the normal range (mean ± SD of ^{3}H-thymidine incorporation: $22 \pm 3 \times 10^3$ cpm) in conventional MLC with T-depleted allogeneic peripheral mononuclear cells from normal subjects.

After 48 hours' culture in presence of 100 IU/mL IFNα, the stimulatory capacity of the hairy cells of both the cases studied was markedly increased (Fig 3), the difference being statistically significant at the lowest cell concentrations. At the highest cell concentrations, the MLR was no longer proportional to the number of stimulator cells added. As shown in Fig 3, at the highest cell concentration, the increase of stimulatory capability after exposure to IFNα was significant only in case 3 hairy cells v T_4 cells.

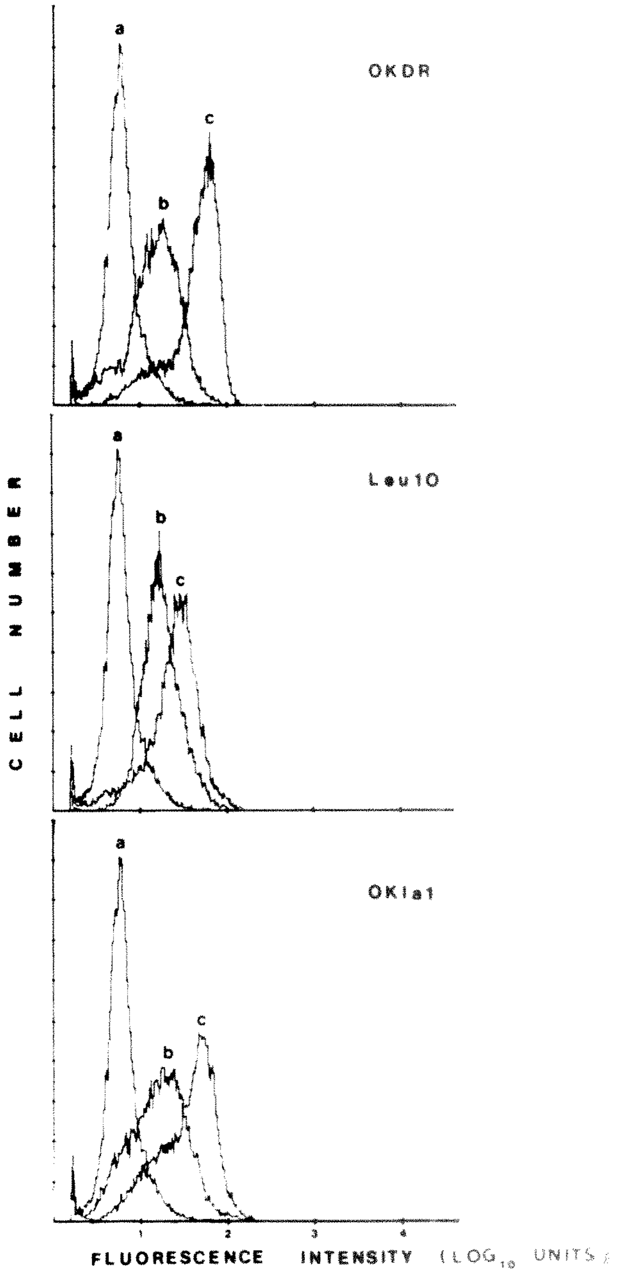

Fig 2. FACS analysis of splenic hairy cells from case 2 with OKDR, OKIa1, and Leu-10. The cells were cultured with or without 100 IU/mL of recombinant IFNα for 48 hours. (A) Control of IFN-treated cells. (B) Non–IFN-treated cells. (C) IFN-treated cells. Settings for FACS 420 were photomultiplier 700 mV, laser 350 mW at 488 nm.

DISCUSSION

We studied the effects in vitro of recombinant IFNα on homogeneous spleen hairy cell populations. For this purpose, immunophenotype analysis was carried out with a large panel of mAbs: the cytofluorimetric pattern of positivities and ultrastructural cell features was then evaluated. In addition, functional behavior in MLC was investigated. In agreement with a recent report by Kosmeyer et al,[10] fresh

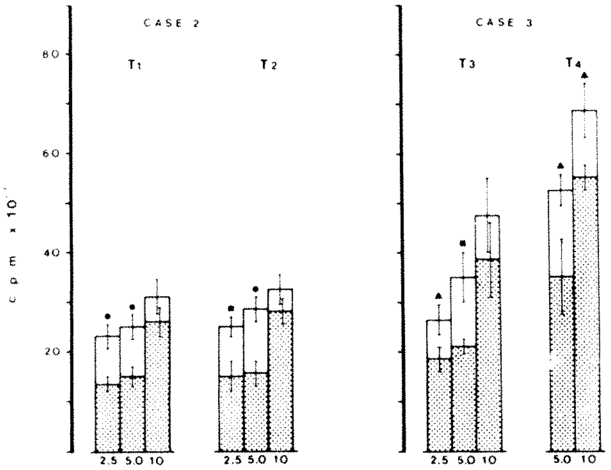

Fig 3. Increase of stimulatory capacity of IFN-treated splenic hairy cells in MLC. Splenic hairy cells were cultured with (□) or without (▨) 100 IU/mL IFN for 48 hours, irradiated with 3,000 rad, and then cocultured at three concentrations (2.5, 5.0, and 10 × 10^4 cells/200 μL as shown on the abscissa) with 10^5 purified T cells from four healthy donors (T_1 through T_4). The mean ± SD of ^{3}H-thymidine uptake of purified T_1 through T_4 cells in conventional MLC with non-T peripheral mononuclear cells from allogeneic donors was 22 ± 3 × 10^3 cpm. Cultures were done in quintuplicate and results are expressed as cpm ± SD. (▲, $P < .05$; ■, $P < .02$; ●, $P < .01$).

hairy cells from the three cases we studied all expressed a typical immunophenotype consistent with their proposed B cell origin (SmIg$^+$, B1$^+$, BA1$^+$, OKIa1$^+$, OKDR$^+$). Positivity for anti-Tac, which binds interleukin 2 (IL 2) receptor, was initially reported in activated and malignant T cells.[16] Recently, however, IL 2 receptor has also been identified on activated B cells,[23] and our samples showed positivity for this mAb. Furthermore, our study confirmed that Leu-M5 and HC2 are useful in identifying cells positive for a B cell-specific antibody (ie, B1) as hairy cells.[14,15]

The expression of class II HLA antigens on the surface of hairy cells was studied with both OKDR (specific for DR monomorphic antigens) and Leu-10 (specific for DR-related DC/DS molecules) in view of the observation that they are expressed differentially on normal hemopoietic and leukemic human cells according to their degree of differentiation.[24] Although the fluorescence intensity for Leu-10 was weaker than in normal peripheral B lymphocytes, we detected both antigens in all the cases, as previously described in Meijer et al's immunohistochemical study.[25] On the contrary, Faille et al[26] were not able to demonstrate DC/DS molecules on isolated cells in one case of HCL using immunoprecipitation techniques; however, they did report the expression of these antigens after exposure of hairy cells to chemical inducers.

In our study, viability, morphology, and cytochemistry of hairy cells were unaffected by culturing. We only observed constant ultrastructural features of increased protein synthesis. As this finding was detected in presence or absence of IFN, we consider it attributable to an aspecific effect of FCS.

Also the immunophenotype was unaffected by culturing apart from the appearance of OKM1 positivity in case 2 irrespective of the presence of IFN. The unchanged morphological and cytochemical features of cultured cells allowed us to exclude a relative increase of monocytes in the culture system. Moreover, OKM1 is not strictly specific for the monocytic lineage,[27,28] and it has already been shown by others to react with both fresh and cultured hairy cells.[29,30]

It is interesting that in this study, human recombinant IFN alpha-2C enhanced the expression of class II HLA antigens in hairy cells. This phenomenon was accompanied by increased stimulatory activity of hairy cells in MLC, functional behavior well known to be closely HLA-DR–linked.[31] This effect was more evident at the lowest hairy cell concentrations, probably due to a functional limit of cultured T cells' responder activity. The increased expression of class II HLA antigens can be attributed to a direct action of IFN, since it was neutralized by the addition of anti-IFNα mAb at the start of culture. No differences were seen when hairy cells were pretreated with anti-Tac; this supports the hypothesis that the IL 2 receptor is not involved. IFNα was active in vitro at a low concentration (20 IU/mL), comparable to that reached in vivo with the administration of therapeutic doses (5 × 10^6 IU/d).

It is known that IFNs can modify the membrane behavior of cells exposed to their action and, particularly, HLA antigen and Fc receptor expressions.[1] In fact, enhancement of class II HLA antigens has been shown on human monocyte cell lines[6] and on various murine cell lines of hemopoietic, lymphoid, macrophage, fibroblast, and neuronal origin[7,9] in presence of recombinant or highly purified IFNγ. An increase of mRNAs codifying these HLA antigens has been demonstrated by other authors in IFN-treated human lymphoblastoid and melanoma cell lines.[32]

The biological meaning of these effects is still discussed. The induction of Ia antigens, particularly on B lymphoma and macrophage cell lines, may result in a functional enhancement of antigen-presenting capability.[9] Another hypothesis is that hemopoiesis may be modulated by IFN through Ia-restricted communication with regulatory T cells.[33,34] Finally, it is conceivable that this class II HLA antigen modulation is an expression of differentiation in normal hemopoietic cells and their neoplastic counterparts. The fact that IFNα and β are less effective in inducing class II HLA antigens[6] than IFNγ, and that the latter is normally released by activated T cells, seems to indicate that IFNγ plays a more important role in the physiologic regulation of these antigens.

Hokland et al,[35] using partially purified IFNα, demonstrated an increase of HLA antigen expression detected by β_2-microglobulin on peripheral blood mononuclear cells and in various human non-T lymphoblastoid cell lines. Nevertheless, in the same experimental conditions, the HLA-DR–related Ia antigens were unaffected. Our study shows that hairy cells are sensitive to IFNα, as indicated by the increase of HLA-DR and DR-linked antigen expression. This sensitivity was particularly notable, since we failed to observe such a phenomenon on cells from four cases of B-CLL. Moreover, in OKDR$^+$ Leu-10$^-$ cells from ALL (two cases of n-ALL, one case of c-ALL), IFNα treatment did not induce either DR antigen expression enhancement or positivity to Leu-10 (data not shown).

IFNα did not seem to have a differentiating action on hairy cells, since we did not observe any other immunocytomorphological modification, indicating cellular maturation. On the other hand, HLA antigens are known to be involved in cell-mediated cytotoxicity v virus-infected and possibly tumor cells. In particular, class II HLA antigens have been implicated in the sensitization phase of cell-mediated cytolysis and as restriction antigens in cytotoxicity mediated by T4$^+$ clones.[36] This cytotoxicity mechanism has been demonstrated also in interactions between cytotoxic effector lymphocyte clones and autologous B lymphoblastoid lines transformed by Epstein-Barr virus.[37] It may be speculated that the selective enhancement of class II HLA antigens shown in our study, by potentiating a cytotoxic mechanism, contributes to the antileukemic activity IFNα exerts in vivo. Our data may therefore help to elucidate the multifactorial mechanism of therapeutic effect of IFNα in HCL.

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DDAVP in Type IIa von Willebrand's Disease

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Christine Jenneau, and Yvette Sultan

1-D-Amino(8-D-arginine)-vasopressin (DDAVP) infusion in three patients with type IIa von Willebrand's disease (vWD) resulted in a normalization of the factor VIII coagulant, factor VIII-related antigen, and von Willebrand factor (vWF) (ristocetin cofactor) activities and the bleeding time. The normalization of these hemostatic parameters persisted for four hours. Over the same time period there was a marked increase in the quantity of the vWF multimers when blood was collected in the presence of protease inhibitors. The vWF multimers present were even larger than the normal. When blood was collected in the absence of protease inhibitors, a smaller increase in the plasma vWF multimers was observed and fewer of the intermediate and larger vWF multimers were seen; multimers larger than those present in normal plasma were not visualized. The platelet vWF multimers and activities did not change with or without inhibitors. These studies suggest that there is a subgroup of patients with type IIa vWD who respond to DDAVP with complete normalization of their hemostatic abnormalities and whose vWF is sensitive to proteolysis.

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1-D-AMINO(8-D-ARGININE)-VASOPRESSIN (DDAVP) has been shown to increase the plasma levels of factor VIII coagulant (VIII:C), factor VIII-related antigen (VIII R:Ag), and factor VIII/von Willebrand factor activity (fVIII/vWF) in normal subjects.[1,2] This synthetic analog of the natural antidiuretic hormone arginine vasopressin has been used successfully in the treatment of bleeding episodes in mild to moderate forms of hemophilia A and in certain types of von Willebrand's disease (vWD).[3-6] In particular, it appears that patients with type I vWD respond quite well to DDAVP infusions with a rise in VIII:C, VIII R:Ag, vWF activity and normalization of the bleeding time, while patients with type IIa vWD have a rise in their VIII:C and VIII R:Ag, but their vWF activity and bleeding time are not corrected. It has been reported that the largest and intermediate-sized multimers do not appear in the plasma of type IIa vWD patients after DDAVP infusion.[7] We administered DDAVP to three patients with type IIa vWD, which resulted in release of the largest vWF multimers, correction of vWF activity, and correction of the bleeding time in each patient. This suggests that DDAVP may be a useful agent in the treatment of some patients with type IIa vWD.

MATERIALS AND METHODS

Patients. Patients described in this study have been diagnosed as type IIa vWD. They have absent ristocetin-induced platelet aggregation and normal or near normal VIII R:Ag levels with marked reductions in vWF activity (ristocetin cofactor) (Table 1). They appear to be similar to the patients described as type IIa by other authors.[8-10] The patients were aware of the experimental nature of the study and gave their informed consent. All experiments were performed according to the declaration of Helsinki. DDAVP (Stimate, Revlon-Armour Berkley Heights, NJ; Minirin, Ferring Pharmaceuticals, Inc, Malmo, Sweden) was administered at a dose of 0.3 μg/kg body weight; the medication was added to 50 mL of normal saline and infused intravenously over a 30-minute period. Before, during, and after the infusion period, blood pressure and pulse were checked every 30 minutes in the contralateral arm. The patients were questioned as to any subjective symptoms during or after the treatment.

Blood. Blood samples were collected by venipuncture with a 19-gauge needle, using a two-syringe technique and polypropylene syringes; the blood in the first syringe was used for complete blood counts and the blood collected in the second syringe was used for coagulation studies. Two sets of tubes were used for the coagulation studies: one set of tubes contained sodium citrate 3.2% (final concentration 10.9 mmol/L) and the second set of tubes contained an identical concentration of sodium citrate with 5 mmol/L EDTA, 1 mmol/L leupeptin, and 6 mmol/L *n*-ethylmaleimide (NEM). The proportion of blood to anticoagulant was the same in both sets of tubes (9:1). The blood was centrifuged at 3,000 g at 4 °C for 15 minutes, and the plasma was separated and tested fresh or was frozen at −70 °C for no longer than seven days before testing. Samples were obtained before and at 30, 60, 120, and 240 minutes in two patients and at 20, 40, 60, 90, 120, 240 minutes, and 24 hours in one patient after infusion.

The VIII:C activity was assayed on fresh samples by a one-stage method based on the partial thromboplastin time as previously described.[11] VIII R:Ag was assayed by electroimmunodiffusion according to Laurell and the vWF activity was measured using formalinized fixed platelets as previously described.[11] The multimeric organization of the vWF was analyzed by glyoxyl agarose electrophoresis in the presence of sodium dodecyl sulfate.[12] The technique was modified from the original description, including a reduction in sample size to 10 μL and a change in gel thickness from 2.0 mm to 0.5 mm. Samples were run at 50 mA until the tracking dye reached 1 cm from the end of the gel. Temperature was maintained at 17 °C throughout the run. The vWF multimers were identified after incubation in the agarose gel with ^{125}I affinity-purified rabbit antihuman vWF antibody and subsequently developed by autoradiography.

Bleeding times were performed using the technique of Ivy using a standardized template technique before the infusion of DDAVP and two hours after infusion was finished.

Before and two hours after completion of the DDAVP infusion, blood was obtained for analysis of platelet vWF. Platelets were separated from plasma proteins on arabinogalactan gradients as previously described.[12] The plasma-free platelets (1×10^6 per microliter) were lysed by Triton X-100 (Sigma Chemical Co, St Louis), and the supernatants of the platelet lysate were analyzed for VIII R:Ag and vWF activities and for multimeric structure by glyoxyl agarose electrophoresis (see above).

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Table 1. Type IIa vWD

Patient	Bleeding Time (min)	VIII:C (%)	VIII R:Ag (%)	vWf (%)	RIPA (mm/min)
1	>20	38	67	20	NA
		35–42	60–72	12–20	
2	>20	38	34	7	NA
		29–42	30–48	5–15	
3	>30	31	44	7	NA
		26–37	44–60	5–12	
Normal	<8	48–155	55–168	59–143	56–76

Normal ranges were derived from 25 healthy subjects. Values for the patients are those obtained before the DDAVP infusion. The values underneath are the range of at least three determinations. The bleeding times have always been greater than 15 minutes. Shown here are the longest bleeding times recorded for each patient. RIPA, ristocetin-induced platelet aggregation at 1.81 mg/mL ristocetin concentration, mm/min; NA, no aggregation.

RESULTS

The three patients had similar coagulation abnormalities before the infusion of DDAVP. In each, the VIII:C was reduced, and in two patients the VIII R:Ag was reduced on the determination before the DDAVP infusion. vWF was severely decreased in all three patients, with the values ranging from 7% to 20% (Table 1). Bleeding times were prolonged (>15 minutes) in all three patients. The multimeric analyses of the plasma vWF protein were abnormal in that they lacked the largest and intermediate-sized multimers and had enhancement of the minor bands (triplet structure) of each multimer series (Fig 1). The platelet vWF multimeric structure revealed a lack of the largest multimers (data not shown).

Thirty minutes after completion of the DDAVP infusion there was a marked rise in the vWF and VIII:C activities and

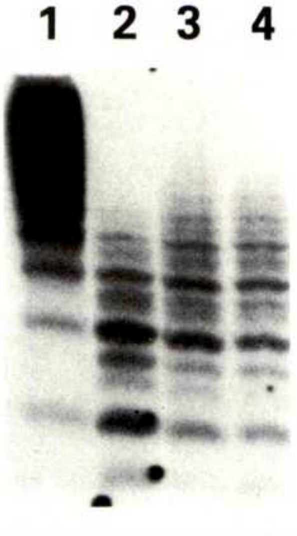

Fig 1. Agarose gel electrophoresis of plasma vWF. Plasma from normal pool (lane 1) and patients 1, 2, and 3 from Table 1 (lanes 2, 3, and 4, respectively) were analyzed by glyoxal agarose gel electrophoresis. Note that the patients lack the intermediate and larger vWF multimers compared with the normal and have increased binding of the radiolabeled antibody to minor bands of each multimer subset. Anode is at the bottom.

Table 2. DDAVP Infusion in Patients With Type IIa vWD

	Patient 1		Patient 2		Patient 3	
	Pre	Post*	Pre	Post	Pre	Post
VIII:C (%)	38	147	38	156	31	115
VIII R:Ag (%)	67	265	34	255	44	238
vWf (%)	20	58	7	72	7	77
Bleeding time (min)	>20	8.5	>15	5	>15	5.5

*Peak of VIII:C, VIII R:Ag, and vWf activity occurred 30 to 60 minutes after DDAVP infusion.

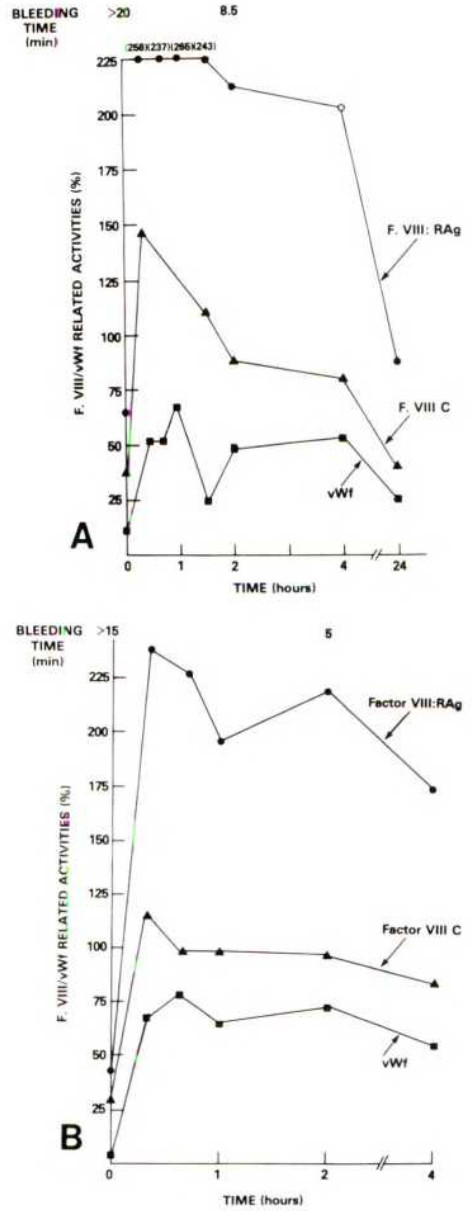

Fig 2. Time sequence of the changes of VIII:C, VIIIR:Ag, and vWf after DDAVP infusion. (A) is Patient 1 and (B) is Patient 2 (Table 1). In both patients the vWf and VIII:C remained in the normal range for up to four hours. In one patient who was studied, the values returned to baseline at 24 hours. In both patients there was normalization of the bleeding time at two hours.

a rise in the VIII R:Ag to normal levels. The levels of all three activities remained normal for at least four hours (Fig 2). The bleeding time, which had been prolonged (>15 minutes) before DDAVP infusion, was corrected at two hours to 5.0 minutes, 5.5 minutes, and 8.5 minutes, respectively, in each patient (Table 2 and Fig 2).

There were marked changes in the patterns of the plasma vWF multimers. This was most apparent in the blood that was collected in the presence of the protease inhibitors (Fig 3). These clearly showed that within 20 minutes after the infusion, there was a marked increase in the amount of antigen present and that larger multimers were present than previously seen in the patients' plasmas; in two out of three cases, the multimers were even larger than those seen in normal plasma. In contrast, blood collected in the absence of protease inhibitors showed only a moderate increase in the intermediate and larger-sized multimers; the largest multimers were not seen. It was also noted that the enhancement of the minor bands of the vWF multimers (seen in baseline studies in the type IIa patients) was less intense in the blood samples collected in the presence of the protease inhibitors. The multimeric structure remained normal at four hours but returned to the preinfusion pattern by 24 hours.

Analysis of the platelet vWF multimers in two of the patients two hours after infusion of DDAVP were similar to those observed before the DDAVP infusion, and the platelet VIII R:Ag and vWF activities did not change significantly two hours after the DDAVP infusion (before infusion, platelet VIII R:Ag 34% and 72%; vWF activity 15% and 6%, respectively; postinfusion, VIII R:Ag 30% and 77% and vWF activity 20% and 9%, respectively).

DISCUSSION

DDAVP has been a useful therapeutic modality for the treatment of patients with mild hemophilia or type I vWD.[3,7] Previous studies using DDAVP in type IIa vWD revealed that despite elevation of the VIII R:Ag and VIII:C to the normal range, the vWF activity and the bleeding time were not corrected, and the multimeric structure of the plasma vWF was virtually unchanged.[7] Contrary to that report, the results in our three patients indicate that at least some patients with type IIa vWD may respond to DDAVP. The increase seen in the VIII R:Ag and vWF activities is paralleled by a change in the multimeric structure of the vWF. When the blood is collected in the presence of protease inhibitors, even larger multimers than those seen in normal plasma are observed after the DDAVP infusion. These data suggest that some patients with type IIa vWD synthesize vWF in their endothelial cells that has a full complement of multimers, and that release of these multimers into the circulation occurs after DDAVP stimulation. Our data with DDAVP infusion indicate that these released multimers are also sensitive to the same enhanced proteolytic digestion as previously described in type IIa vWD.[9]

These studies are in agreement with our previous studies reporting the full complement of multimers in patients with type IIa vWD when their blood is collected in protease inhibitors.[9] Our data are also compatible with observations by McCarroll et al[13] showing that DDAVP increases the vWF antigen II in type IIa vWD in a manner similar to normals and patients with type I vWD. Recent observations indicate that vWF antigen II and vWF are both synthesized in endothelial cells and form a single protein complex.[14] The separation of the vWF antigen II from the vWF molecule results from proteolysis. It is not clear where and how the proteolysis of the vWF occurs in these patients, but it does appear to affect both the vWF antigen II and the vWF.

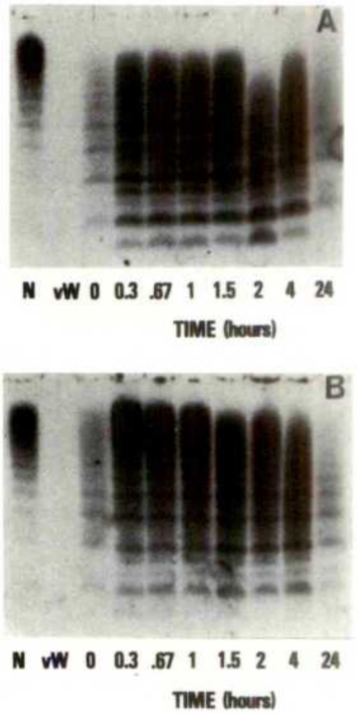

Fig 3. Glyoxyl agarose gel electrophoresis. On the left (A) are the patients' plasma samples before and after DDAVP infusion from which blood was collected in sodium citrate without protease inhibitors. On the right (B) are plasma samples obtained at the same time, from blood collected into tubes containing sodium citrate and EDTA, leupeptin, and NEM. N is normal plasma; vWD is from the patient with severe vWD with <3% factor VIII-related activities. The origins are visible at the top of the figure. The plasmas collected in the presence of protease inhibitors show larger multimeric structures at the early time points after DDAVP infusion than do the plasmas collected in the absence of protease inhibitors. Also, the minor bands of the major multimers are much less prominent than those seen in the plasma from blood collected in the absence of protease inhibitors.

Thus this study suggests that some type IIa vWD patients do synthesize a full complement of multimers and do release them after DDAVP infusion. In these patients, DDAVP infusion may be a useful substitute for cryoprecipitate infusion, thus avoiding the risks associated with infusion of plasma products.

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A New Gene Deletion in the α-Like Globin Gene Cluster as the Molecular Basis for the Rare α-Thalassemia-1 ($--/\alpha\alpha$) in Blacks: HbH Disease in Sickle Cell Trait

By Martin H. Steinberg, Mary B. Coleman, Junius G. Adams III, Robert C. Hartmann, Hussein Saba, and Nicholas P. Anagnou

A novel deletion of at least 26 kilobase of DNA, including both α-globin genes, the $\psi\alpha$- and $\psi\zeta$-globin genes, but sparing the functional ζ-gene was found in a 10-year-old black boy with HbH disease and sickle cell trait. This particular deletion has not previously been described in blacks. Its existence makes it likely that the absence of Hb Barts hydrops fetalis in blacks is due to the rarity of the chromosome lacking two α-globin genes rather than a result of early embryonic death due to the failure to synthesize embryonic hemoglobins because of deletion of functional ζ-globin genes.

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α-THALASSEMIAS are most commonly a result of deletions of α-globin genes.[1,2] Four clinical syndromes are recognized: the silent carrier, with loss of a single α-gene ($-\alpha/\alpha\alpha$); α-thalassemia trait, in which two genes are missing, by loss of either both α-genes from a single chromosome ($--/\alpha\alpha$) or one gene from both chromosomes ($-\alpha/-\alpha$); HbH disease, which is the compound heterozygous state for the single and double deletion chromosome ($--/-\alpha$); and hydrops fetalis, in which no α-genes are present ($--/--$).[3] Varying extents of DNA deletion[4-10] and the existence of nondeletion types of α-thalassemia[11-17] add to the complexity of these disorders. The ($-\alpha/$) chromosome is a common genetic lesion in blacks,[5,6] yet HbH disease is seldom found[18] because of the rarity in this population of a chromosome lacking both α-globin genes ($--/$). We present studies of a new deletion, producing a ($--/$) chromosome, found in a black child with HbH disease. Sickle cell trait (HbAS) was also present in this patient. The interaction of HbAS and HbH disease resulted in a distinct phenotype.

MATERIALS AND METHODS

Blood counts and erythrocyte indices were obtained using a Coulter electronic cell counter (Hialeah, Fla). Hemoglobin electrophoresis was performed on polyacrylamide gels, cellulose acetate membranes, and citrate agar gels.[19] The hemoglobin fractions were quantified after their elution from polyacrylamide gels. HbF was measured by alkali denaturation.[20] HbH detection in the erythrocytes was done using brilliant cresyl blue. The relative rates of α- and non-α-globin synthesis were measured after incubation of peripheral blood with [^{3}H-]-leucine and the separation of globin chains by CMC column chromatography,[21] as previously described.[22] The proportion of β^S synthesized was calculated as the $\beta^S/\beta^A + \beta^S$ ratio.

Restriction endonuclease mapping of α- and ζ-globin genes was done by Southern blotting,[23] as previously described.[24,25] High-molecular-weight genomic DNA extracted from white blood cells was digested with the restriction enzymes *Eco*RI, *Bam*HI, *Bgl*II, *Kpn*I, *Sac*I, *Hpa*I, and *Hind*III, according to directions of the manufacturer, and then electrophoresed for 18 hours in 0.8% agarose gels using a submerged horizontal gel apparatus. The probes used were the nick translated plasmid JW101[26] for α-globin genes and the 1.8 kilobase (kb) *Sac*I fragment from plasmid pBR31ζ_1 for the ζ-globin genes.[25]

RESULTS

The family pedigree is shown in Fig 1. The proband was a 10-year-old black male, who was referred because of mild anemia and microcytosis that was discovered when the patient was being evaluated for a tonsillectomy. There were no abnormalities detected on physical examination. Growth and development were normal, and he was of above-average intelligence.

Hematologic data are shown in Table 1. The blood film of the proband showed hypochromia, microcytosis, anisocytosis, poikilocytosis, and the presence of target cells. The HbA$_2$ and HbF levels were normal in all individuals, and the proband had a normal serum iron and total iron binding capacity. His reticulocyte count was 1.8%. HbH (β_4) and Hb Barts (γ_4) were not detected after electrophoresis on cellulose acetate membranes or polyacrylamide gels. No HbH inclusions were generated after incubation of the proband's fresh cells with brilliant cresyl blue. Erythrocytes of the proband contained 25% HbS, while his reticulocytes, on incubation with [^{3}H]-leucine (Table 1 and Fig 2), synthesized 25% β^S globin. The α-non-α ratio was 0:60.

Results of restriction endonuclease mapping studies of the α- and ζ-globin genes of the proband, some family members, and controls are shown in Figs 3 and 4. Digestion with *Eco*RI and *Bgl*II and hybridization with the α-probe showed a single abnormal 19-kb and 16-kb fragment, respectively, in the proband (Fig 3). Furthermore, double digestion with *Hind*III and *Bgl*II generated a 10.5- and 4.5-kb fragment only with the absence of the normal 2.0- and 1.7-kb fragments. The presence of the abnormal *Eco*RI and *Bgl*II fragments, along with the absence of the 2.0- and 1.7-kb *Hind*III-*Bgl*II fragments, is consistent with a ($-\alpha/$) chromosome, resulting from the "rightward" (-3.7 kb) deletion common in blacks. This chromosome apparently was transmitted to the proband from his mother (I-2), who is a heterozygote for α-thalassemia-2 ($-\alpha/\alpha\alpha$). The absence of normal bands in the *Eco*RI, *Bgl*II, and *Hind*III plus *Bgl*II

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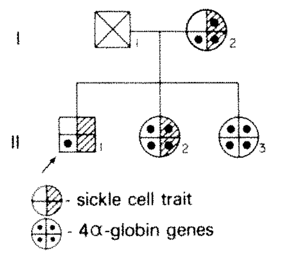

Fig 1. Family pedigree. The father I-1 was not available for study.

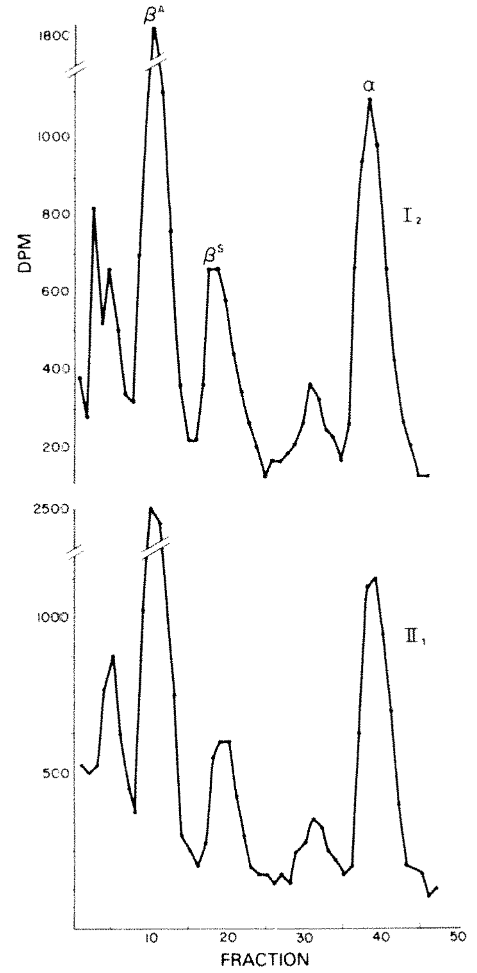

Fig 2. Globin biosynthesis studies of the proband, II-1, and mother, I-2. There was a deficit of α-globin synthesis relative to non-α-globin. This was more severe in the proband, who also had a reduction in β^S synthesis.

digests in the proband is consistent with either a $(-\alpha/-\alpha)$ or a $(--/-\alpha)$ genotype.

To explore further the characteristics of the $(--/)$ chromosome, and to document the presence of HbH disease, DNA was digested with *Bgl*II, *Eco*RI, *Hind*III, *Bam*HI, *Hpa*I, *Kpn*I, and *Sac*I and was hybridized to a specific ζ-globin probe (Fig 4). Abnormal fragments were detected with *Bgl*II, *Eco*RI, and *Hind*III digestions. The 16-kb *Bgl*II and 19-kb *Eco*RI fragments are the common fragments detected previously with the α-probe and corroborates the rightward (−3.7) deletion present in one chromosome of the propositus (P) and his mother (M). The abnormal 8.5-kb *Bgl*II, 7.2-kb *Eco*RI, and ~23.0-kb *Hind*III fragments indicate that these sites 3′ to the functional ζ-gene have been removed by the deletion on the other chromosome of the proband (Fig 4). Furthermore, digestion with *Bam*HI, *Hpa*I, and *Sac*I revealed no abnormal fragments. The reduced intensity of the signal from the 11-kb *Bam*HI fragment, when compared with the 5.9-kb fragment, is consistent with the loss of the *Eco*RI site 3′ to the ζ-gene, as discussed earlier, but does not prove the absence of the *Bam*HI site 2 kb 3′ to the ζ-gene. Thus the 5′ end point of the deletion can be localized between the *Eco*RI site at 14 kb in Fig 4 and the *Bam*HI site immediately after the 3′ end of the ζ-gene (Fig 4). Because the 1.8-kb *Sac*I band actually represents comigration of three fragments (two of which are included in the deletion), it is difficult to ascertain the differences in the intensity of the 1.8-kb fragment between the normal and the patient. Thus a region of uncertainty of about 1 kb between the *Sac*I and *Eco*RI sites exists (Fig 4). Based on the data of the α- and ζ-fragments, the 3′ end of the deletion must include at least the *Hind*III, *Kpn*I, and *Bgl*II sites located downstream from the α-gene cluster (Fig 4). Digestion with *Kpn*I and hybridization with the ζ-probe showed a doublet of at least 25 kb and 19 kb and an 11-kb fragment. The 19-kb fragment appears to be from the chromosome containing the single α-gene deletion. The ~25-kb fragment may represent an abnormal band caused by the large deletion, as it appears different from the ~23-kb fragment in the normal control. Multiple digestions of different DNA samples with *Hpa*I failed to reveal an abnormal-sized fragment. This may be due to either an abnormal fragment that fortuitously is nearly identical in size to the normal fragment or an abnormal fragment so large that it fails to transfer. Due to the lack of suitable probes for DNA 3′ to the α-genes, no localization can be made regarding the exact 3′ end point of deletion. Thus the deletion must be at least 26 kb long.

Table 1. Hematologic Data

	I-2	II-1*	II-2	II-3	Control
Hemoglobin (g/dL)	13.4	10.1	12.8	—	13.6
MCV (fL)	78.0	53.0	86.0	—	98.0
MCH (pg)	26.3	16.8	28.8	—	33.0
HbA (%)	61.2	71.6	59.2	97.2	97.4
HbS (%)	35.9	25.0	37.7	—	—
$\alpha/\beta^A + \beta^S$ ratio	0.76	0.60	0.88	1.0	0.95
β^S synthesized (%)	35.0	25.0	35.0	—	—

*Proband.

HLA typing and studies of erythrocyte antigens showed the proband to be HLA A-28,30, B-53,5 like, C-1,4, BW-4,6, DR-2,6Y, MT-(1,2) and to be blood group O, D+C−E−c+e+; M+N−S−st; P_1,+; K−k+; Fy (a−b−); Jk (a+b−); Le (a−b+).

DISCUSSION

Deletions other than the small rightward and leftward deletions[27] have been described that have removed both, or most of both, α-globin genes from a single chromosome. In

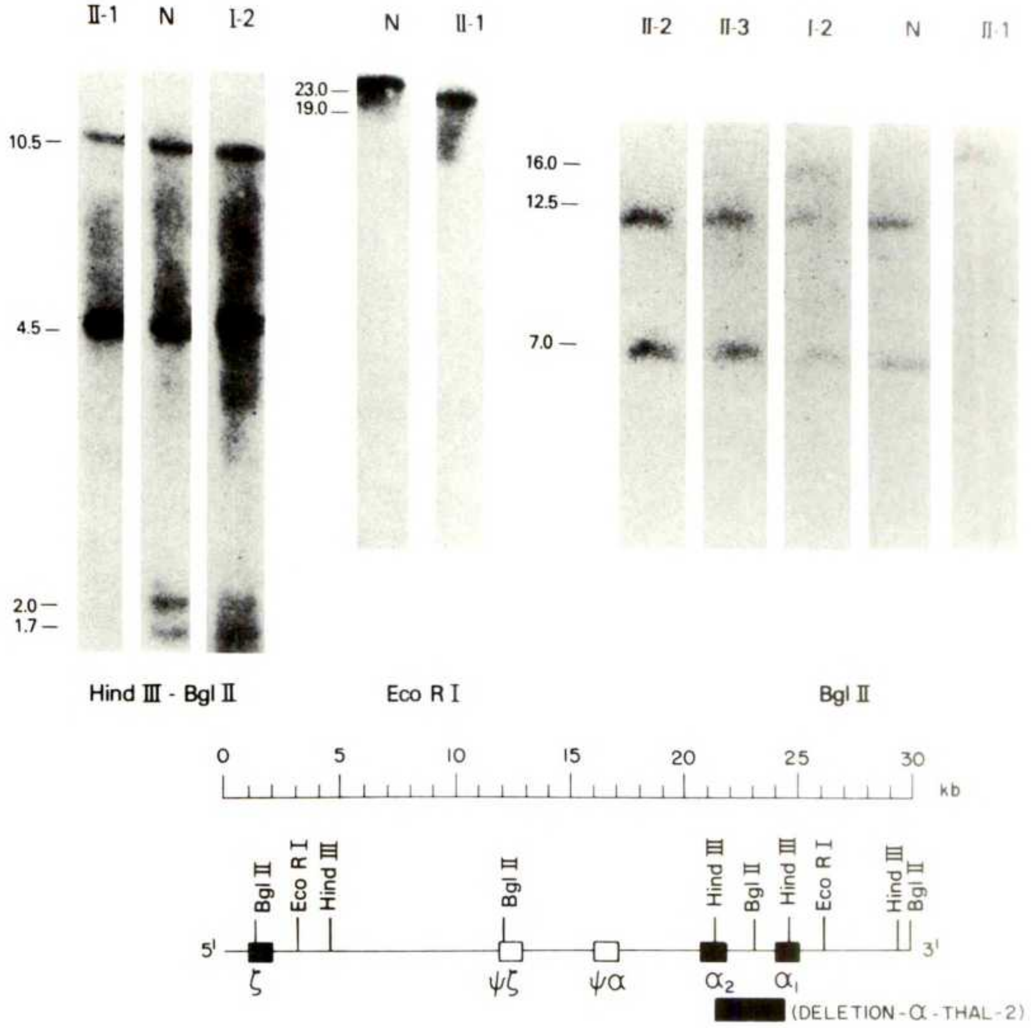

Fig 3. Southern blot analysis of the α-globin using the α-specific plasmid JW101. The restriction map shows only the sites explored with the enzymes *Eco*RI, *Bgl*II, and *Hind*III. Black boxes represent functional genes and white boxes the pseudogenes. The α-thalassemia-2 deletion is depicted as a black bar.

Italians and Turks, an extensive deletion involving at least 25 kb of DNA, including the 5′ portion of the α_1-gene and extending 5′ to the ζ-gene, has been described.[8] In Greeks and Cypriots, two deletions have been reported: a 5.2-kb deletion, including the α_2-gene and 5′ portion of α_1, leaving intact the ψζ- and ζ-genes[7]; and a 17.4-kb deletion, removing the 5′ part of α_1, α_2, ψα, and ψζ but preserving the ζ-gene.[9] From Thailand, a (− −/) chromosome lacking ψα, α_2, and α_1 but preserving ψζ and ζ was described by Pressley et al.[9] Deletion of the ζ-, ψζ-, ψα-, and both α-genes has also been described by Weatherall et al[28] in a north European boy with HbH disease and mental retardation. Embury et al[29] have described (− −/) chromosomes in two black patients with intact ζ-genes, but the full details of the extent of the deletions have not yet been reported. Felice et al[10] recently reported an extensive deletion of the entire α-gene cluster in a black patient with HbH disease and HbAS. This was the first well-characterized (− −/) chromosome of blacks described. In our patient, the deletion extends past the ψζ-gene but stops short of the functional ζ-gene. This might preserve in utero expression of the ζ-globin gene, although we have no direct evidence that this is so. Absence of the functional ζ-gene as a result of a deletion, such as that described by Felice et al,[10] had been postulated to underlie the rarity of the Hb Barts hydrops fetalis syndrome in blacks, as the lack of embryonic hemoglobins, Gower 1 ($\zeta_2\epsilon_2$), and Portland ($\zeta_2\gamma_2$), would lead to early embryonic death.[29] In neither our case nor that of Felice et al[10] was the father of the proband available for study. While both fathers were black, Caucasian genes may constitute about 10% of the southern US black gene pool,[30] although estimates range as high as 35% in other black populations.[31,32] The D+C−E−c+e+ (Rh^o/Rh^o) and Fy(a−b−) phenotype of our patient provides little indication of Caucasian ancestry.[30]

The (− −/) chromosomes in blacks, as in other ethnic groups, results from more than one type of deletion,[10,29] some of which spare the functional ζ-globin gene. The absence of hydrops fetalis in blacks more likely is a result of the rarity of the (− −/) chromosome,[5,6] rather than the loss of the functional ζ-globin gene from some of these chromosomes.

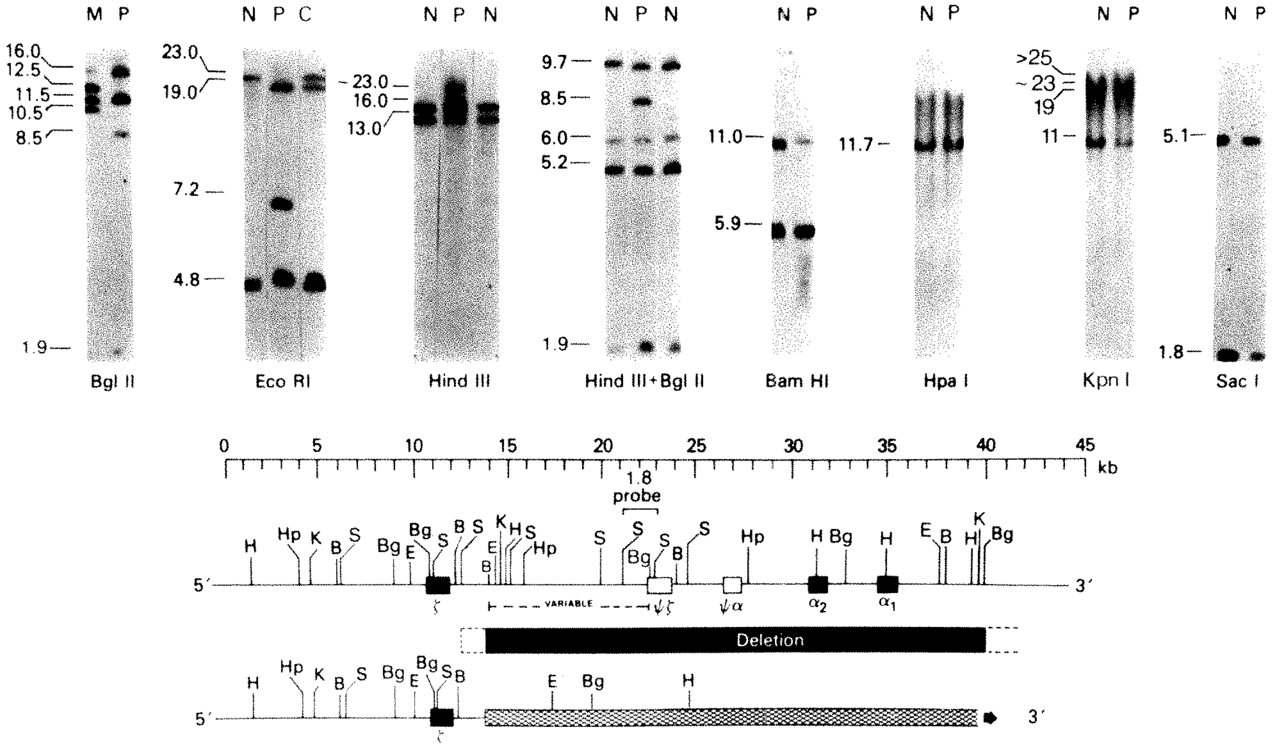

Fig 4. Southern blot analysis of the ζ-globin region using the 1.8-kb *Sac*I fragment, which contains the 5′ end of the ψζ-gene. Because ψζ- and ζ-genes are highly homologous, the probe detects fragments generated from both genes equally well. The top restriction map represents the restriction sites and the chromosomal order of the genes in the normal α-like globin gene cluster. Black boxes indicate the functional genes, white boxes, the pseudogenes. The highly variable region between the ζ- and ψζ-genes is indicated by broken lines.[39] The extent of the deletion is depicted as a black bar. The lower diagram represents the DNA rearrangement in the abnormal chromosome resulting from the deletion. The DNA sequences from the 3′ end of the deletion containing the new *Eco*RI, *Bgl*II, and *Hind*III sites brought into the vicinity of the ζ-gene region, are shown as a hatched bar. For comparative reasons, a control in the *Eco*RI digest (C) was an α-thalassemia-2 deletion exhibiting the normal 23- and the abnormal 19-kb fragments. Abbreviations for the restriction enzyme sets: Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; B, *Bam*HI; Hp, *Hpa*I; K, *Kpn*I, S, *Sac*I; M, mother; P, propositus; N, normal control.

This is the fourth patient reported with a combination of HbH disease and HbAS.[3,10,31] One individual was North African.[3] In all, the hematologic picture was similar; there was marked microcytosis, HbS levels that were lower than those seen in uncomplicated HbAS and even lower than in HbAS with an $(-\alpha/-\alpha)$ genotype, very low levels or absence of HbH, and a marked reduction of α-globin synthesis. The mechanisms by which these α- and β-globin disorders modulate the expression of each other have been previously discussed.[10,33,34] The reduction in the porportion of HbS is a result of β^A-globin competing more efficiently than β^S-globin for the limited amounts of α-globin chain.[34,35] While HbH disease in blacks characteristically exhibits lower levels of HbH than this disorder in Asians,[3,8,36-38] the near absence of β_4 in the presence of HbAS is likely a result of more efficient HbA formation in addition to the presence of only a single β^A-allele and the instability of a β^S homotetramer.

NOTE ADDED IN PROOF

Higgs et al[40] have described an α-globin gene deletion similar to the one in this report in British individuals with only slight differences in the sizes of the abnormal restriction fragments.

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Lymphoblastic Lymphoma: An Immunophenotype Study of 26 Cases With Comparison to T Cell Acute Lymphoblastic Leukemia

By Lawrence M. Weiss, Jane M. Bindl, Vincent J. Picozzi, Michael P. Link, and Roger A. Warnke

A series of 26 lymphoblastic lymphomas (LLs) and 13 T cell acute lymphoblastic leukemias (ALLs) were investigated using a battery of monoclonal antibodies applied to tissue frozen sections. Twenty-one of the LLs were of T lineage. All but one of the T cell LLs were of immature thymic phenotype, mostly corresponding to stage II cortical thymocyte development. The T cell LLs expressed Leu-1 in 100%, Leu-4 and Leu-9 in 95%, and Leu-5 in 85% of the cases. The high percentage of Leu-4 expression in this series is probably due to detection of cytoplasmic antigen with our methods. One LL was of pre-B or B cell and two cases were of common ALL phenotype. Two cases were of undefined phenotype, expressing markers of both B and T cell differentiation. Pediatric cases showed a greater tendency toward T cell phenotype than did adult cases. The cases of T cell ALL were immunophenotypically similar to the cases of T cell LL but showed a tendency toward a more immature phenotype.

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LYMPHOBLASTIC LYMPHOMA (LL) is a neoplasm recently redefined as a distinct clinicopathologic entity by Barcos and Lukes.[1] These investigators proposed that LL was a lymphoma of T cell type, and since that time, many workers have come to regard LL as the lymphomatous variant of T cell acute lymphoblastic leukemia (ALL), due to the similarity of cytologic features and the apparent overlap of clinical features.[2] Immunologic studies of T cell ALL have shown a heterogeneity of immunophenotypes that can often be correlated to stages of T cell development.[3-5] These studies have also compared T cell LL with T cell ALL and demonstrated that the cases of T cell LL often show immunologic markers corresponding to a later period of development of maturing thymocytes than do those of T cell ALL.[4,5] These papers have largely concentrated on the T cell LL, and with the exception of the work of Kaneko et al[6] and Cossman et al,[7] little data exist on the proportion of LLs that are of T cell origin. This information is important, as demonstration of T cell origin may prompt clinicians to select alternative therapeutic regimens.[8] In Cossman et al's study,[7] eight of 11 cases were shown to be T cell immunophenotypes, although only eight of these cases met the more rigorous criteria of less than 25% lymphoblasts in the bone marrow set by others.[4] In addition, Cossman et al found a high percentage of cases (45%) with helper or cytotoxic/suppressor phenotype; they found no cases with cells expressing both helper and cytotoxic/suppressor antigens in contrast to the work of other authors.[4,5,7,9] For example, in the study of Roper et al,[5] only five of 16 cases showed either helper or suppressor phenotype, whereas seven cases showed expression of both T4 and T8. In Kaneko et al's study,[6] five of nine cases were found to be of T lineage. However, most of the cases in this series were investigated using rosetting techniques and only two of the cases were studied using monoclonal antibodies.

In view of the paucity of immunologic data available on LL and the apparent discrepancies among the previous reports, we undertook an immunophenotypic study of LL using a battery of 20 monoclonal antibodies applied to frozen tissue sections. In addition, we examined cases of T cell ALL

Table 1. Monoclonal Antibodies Used

Antigen/Clone/Similar Antigens	Predominant Expression
Leu-1/L17F12*/T1,T65	T cells (and some B cell lymphomas)
Leu-2/SK1*/T5,T8	Cytotoxic/suppressor T cells
Leu-3a/SK3*T4	Helper T cells, macrophages, Langerhans cells, dendritic reticulum cells
Leu-4/SK7*/T3	T cells
Leu-5/ATM1.1*/T11/Lyt-3	T cells (sheep erythrocyte receptor)
Leu-6/SK9*/T6	Langerhans cells and some cortical thymocytes
Leu-9/4H9†/3A1, WT1	Cytotoxic/suppressor and most helper T cells
Ia/L203†	B cells, macrophages, activated T cells, Langerhans cells
CALLA/J5‡	Most non-T, non-B ALL; some lymphomas
T9§	Cells expressing transferrin receptor
T10§	T cells and plasma cells
Mu†	B lymphocytes and plasma cells
B1‡	B lymphocytes, some monocytes/macrophages
B2‡	Cells expressing C3d receptor
T200/L3B12	Panleukocyte
Ki-67‖	Proliferating cells
Leu-15*	NK cells, suppressor T cells, neutrophils, eosinophils, monocytes
T05‖	Cells expressing C3b receptor
Leu-M1/MMA*	Myeloid cells, some monocytes and macrophages, myelogenous leukemia
Leu-M3/MOP-9*	Monocytes, macrophages, Langerhans cells, and certain endothelial cells

*Becton Dickinson, Mountain View, Calif (Leu-5b was occasionally substituted for Leu-5).

†Dr R. Levy, Stanford, Calif.

‡Coulter Clone, Hialeah, Fla.

§Ortho Pharmaceuticals, Raritan, NJ.

‖Dr D.Y. Mason, Oxford, England.

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using the same methods to provide a comparison between T cell ALL and T cell LL.

MATERIALS AND METHODS

From 1978 to 1984, the Laboratory of Immunopathology at Stanford University Medical Center has obtained fresh frozen tissue from 29 cases of LL, of which 26 yielded adequate specimens for immunophenotypic analysis (12 cases were referred from institutions of the Pediatric Oncology Group as part of a protocol studying immunologic and other biological features of childhood non-Hodgkin's lymphomas). One of these excluded cases was shown to be of pre-B immunophenotype by cell suspension studies.[10] The presenting site of disease was the mediastinum in 15 cases, peripheral lymph nodes in nine, the nasopharynx in one, and the testis in one. The tissue taken for immunophenotyping studies came from lymph nodes in 12 cases, the mediastinum in 11, the nasopharynx in two, and soft tissue in one. In 21 of the 26 cases (81%), the tissue taken for immunophenotype represented the original diagnostic material. Cases were diagnosed as LL on the basis of routine histologic sections using standard criteria[11] and if the bone marrow examination showed less than 25% blasts. In all cases except case 3 (3% blasts), case 23 (5%), and case 25 (20%), the bone marrow was uninvolved. This restrictive definition of LL was chosen to provide a comparison to previous series using similar criteria. In fact, several excluded patients were regarded clinically as having LL, but for the purposes of the study were regarded as ALL due to >25% bone marrow involvement. For comparison, 13 of 14 cases of T cell ALL yielded adequate specimens for study over the same time period.

For the immunologic studies, the cases were frozen, processed, and stained using an avidin-biotin detection system, as previously described.[12,13] Briefly, the procedure consists of a first-stage incubation with one of the monoclonal antibodies listed in Table 1. After washing, a biotin-conjugated horse antimouse antibody is applied before a third stage of avidin-conjugated horseradish peroxidase (Vector Labs, Inc, Burlingame, Calif). The sections are then incubated with diaminobenzidine, followed by copper sulfate, and counterstained with methylene blue.

Monoclonal antibody staining in a case was regarded as 2^+ when more than 50% of cells stained with at least moderate intensity, 1^+ when 10% to 50% stained or the staining was of weak intensity, and as negative when 10% of the cells or less stained (due to possible confusion with residual nonneoplastic populations).

RESULTS

Lymphoblastic lymphoma. The results are tabulated in Table 2. Twenty-one of the 26 cases (cases 1 through 21) were found to be of definite T cell immunophenotype as shown by reactivity with antibodies against at least two of the pan-T cell markers used (Leu-1, -4, -5, and -9). All of these cases tested showed the presence of Leu-1 and only one case each did not express Leu-4 or Leu-9. Leu-5 expression was seen in 17 of 20 (85%) cases tested. The T9 antigen was seen in all cases tested, and T10 expression was seen in eight of 18 cases tested. Ia and CALLA expression were seen in four and eight of these cases, respectively. None of the T cell cases expressed B1, B2, or mu. The T cell cases could be subdivided into three major groups on the basis of Leu-2 and Leu-3 expression. Nine cases showed no expression of Leu-2 or Leu-3. This group lacked expression of Leu-6 in five of eight cases tested. Ten cases in the second group expressed

Table 2. Immunologic Studies of Lymphoblastic Lymphoma

Case No	Leu-1	Leu-2	Leu-3	Leu-4	Leu-5	Leu-6	Leu-9	Ia	CALLA	T9	T10	Mu	B1	B2	L3B12	Ki-67	Leu-15	TO6	Leu-M1	Leu-M3
1	2+	—	1+	1+	1+	—	2+	2+	—	2+	2+	—	—	—	2+	—	—	—	—	—
2	2+	—	2+	2+	2+	1+	2+	—	1+	2+	2+	—	ND	ND	2+	—	—	—	—	—
3	2+	1+	2+	2+	2+	2+	1+	—	—	1+	—	—	—	ND	2+	1+	—	2+	—	—
4	2+	2+	2+	2+	2+	2+	2+	—	—	2+	—	—	ND	ND	2+	—	—	—	—	—
5	2+	2+	2+	2+	2+	2+	2+	—	ND	ND	ND	—	ND	ND	2+	1+	—	ND	—	—
6	2+	2+	2+	2+	2+	2+	2+	—	1+	2+	—	—	—	ND	2+	2+	—	—	—	—
7	2+	2+	2+	2+	2+	2+	2+	—	2+	2+	—	—	—	ND	2+	—	—	—	—	—
8	2+	2+	2+	2+	2+	2+	2+	—	1+	1+	1+	—	—	ND	2+	ND	ND	ND	ND	ND
9	2+	1+	2+	2−	1+	2+	1+	—	2+	2+	2+	—	ND	ND	2+	2+	—	—	—	—
10	2+	2+	2+	—	2+	2+	2+	—	1+	ND	ND	ND	—	—	2+	—	—	—	—	—
11	2+	1+	2+	2−	2+	—	2+	—	—	2+	1+	—	ND	ND	2+	2+	—	—	—	—
12	2+	1+	1+	2−	2+	—	2+	—	—	1+	—	—	—	—	2+	—	—	—	—	—
13	2+	—	—	2−	—	1+	2+	—	—	2+	2+	—	ND	ND	2+	2+	—	—	—	—
14	2+	—	—	2−	2+	1+	2+	2+	1+	2+	2+	—	—	—	2+	ND	2+	—	—	—
15	2+	—	—	2−	1+	2+	2+	—	1+	2+	1+	—	—	—	2+	1+	—	1+	—	—
16	2+	—	—	2−	ND	ND	ND	—	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
17	2+	—	—	2−	2+	—	—	—	—	ND	—	—	ND	—	2+	—	—	—	—	—
18	2+	—	—	2+	2+	—	2+	—	—	2+	—	—	ND	—	2+	1+	—	—	—	—
19	2+	—	—	2+	2+	—	2+	—	—	2+	—	—	—	ND	2+	1+	—	—	—	—
20	2+	—	—	1+	—	—	2+	1+	—	2+	—	—	ND	ND	2+	1+	—	—	—	—
21	1+	—	—	2+	—	—	2+	2+	—	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	—
22	—	—	—	—	—	1+	2+	2+	1+	2+	2+	—	1+	2+	2+	1+	2+	—	—	—
23	—	—	—	—	—	1+	—	2+	2+	1+	2+	—	1+	2+	2+	2+	2+	—	—	—
24	—	—	—	—	—	—	—	2+	2+	1+	1+	—	—	2+	1+	1+	—	—	—	—
25	—	—	—	—	—	—	—	2+	2+	—	—	—	—	—	—	1+	ND	ND	ND	ND
26	—	—	—	—	—	—	—	2+	2+	2+	2+	2+	2+	—	2+	2+	—	—	—	—

ND, not done.

Table 3. Lymphoblastic Lymphoma: Age v Immunophenotype

	T Cell	Indefinite	Common ALL	Pre-B/B Cell
Pediatric (<21)	18	1	1	0
Adult (>21)	3	1	1	1

both Leu-2 and Leu-3. In most of the cases in this group, expression of Leu-6 was also seen (eight of ten cases tested). In the third group, cases 1 and 2, a mature helper phenotype was observed (Leu-2^- Leu-3^+). Leu-6 expression was seen in one of these two cases.

One case (case 26) was found to be of pre-B or B cell type with demonstration of mu chains as well as expression of the B1 antigen. This case was unreactive for all pan-T markers as well as Leu-2, -3, and -6; this case showed expression of Ia, CALLA, T9, and T10.

Two cases (cases 22 and 23) were shown to be of common ALL phenotype. Both cases were CALLA and Ia positive but unreactive for all T cell antigens as well as mu, B1, T9, and T10. B2 reactivity was seen in one of the two cases.

Two cases (cases 20 and 21) expressed an unusual phenotype with markers suggesting differentiation toward both T and B lineage. Case 20 showed reactivity with antibodies against Leu-6 and Leu-9 but no reactivity with antibodies against the other T cell markers Leu-1 through Leu-5, while case 21 showed only expression of Leu-6 among all the T cell markers tested. Both cases showed weak staining for B1 and stronger staining for B2 (C3d), although no staining for cytoplasmic mu was found. Both cases also expressed Ia, CALLA, T9, and T10.

Among the other monoclonal antibodies used in the study, the panleukocyte antibody L3B12 showed reactivity in all but one case. The antiproliferative antibody Ki-67 showed a wide variability in staining, from less than 10% of cells in a case staining to close to 100% with no correlation with immunophenotype. Staining for T05 (C3b) and Leu-15 was seen in two and three cases, respectively, each, while no cases stained with antibodies against Leu-M1 and Leu-M3.

As seen in Table 3, the pediatric cases were almost all T cell immunophenotypes, whereas one half the adult cases were T cell type.

T cell acute lymphoblastic leukemia. The results are tabulated in Table 4. All cases tested reacted with antibodies against Leu-4 and Leu-9. Two of 13 cases failed to express Leu-1, while five of 11 cases failed to express Leu-5. As in the T cell LLs, the T cell ALL cases could be separated into three groups based on Leu 2- and Leu-3 expression, with seven cases failing to express Leu-2 or Leu-3, three cases coexpressing Leu-2 and Leu-3, and three cases showing a Leu 2^- Leu 3^+ mature helper phenotype. As seen in Table 5, the immunophenotypes of T cell ALL and LL were somewhat similar but with the largest category of ALL cases Leu $2^-3^-6^-$ (or Reinherz et al[3] intrathymic stage I) while the largest category of LL was Leu $2^+3^+6^+$ (Reinherz et al[3] intrathymic stage II). Similar to LLs, the T cell ALLs uniformly expressed T9, while T10 expression was seen in six of nine cases tested. Ia and CALLA expression was seen in three and five cases, respectively. Interestingly, one case showed expression of B2. L3B12 showed uniform strong staining in all cases, while Ki-67 demonstrated a wide range of positivity. One case each showed staining for T05 and Leu-M3, while no cases expressed Leu-15 or Leu-M1.

DISCUSSION

Our study confirms the findings of Cossman et al[6] and Kaneko et al[7] that LL comprises tumors of non-T as well as T cell phenotype. We found that the T cell phenotype is by far the most common phenotype; in our series, 81% were definitely T lineage. Only one case was found to be of pre-B or B cell phenotype and only two cases were found to be of the common ALL type. Although the number of adult cases was small in our series, these cases tended to have non-T phenotypes (three of six *v* two of 20 for the pediatric group). One may be able to suspect a non-T cell phenotype on clinical grounds; in a previous series of pediatric non-Hodgkin's lymphomas and in the report of Cossman et al,[7,14] none of the non-T cell LLs had a mediastinal mass. In the current series, none of the B cell or common ALL cases were associated with a mediastinal mass; this presentation appears to be specific for the T lineage cases.

Our study also demonstrates clear differences in immuno-

Table 4. Immunologic Studies of T Cell Acute Lymphoblastic Leukemia

Case No	Leu-1	Leu-2	Leu-3	Leu-4	Leu-5	Leu-6	Leu-9	Ia	CALLA	T9	T10	Mu	B1	B2	L3B12	Ki-67	Leu-15	T05	Leu-M1	Leu-M3
1	1+	—	2+	2+	2+	—	2+	2+	2+	2+	—	—	ND	ND	2+	1+	—	—	—	—
2	1+	—	2+	2+	—	—	2+	—	1+	2+	1+	—	—	—	2+	1+	—	—	—	—
3	2+	—	1+	2+	ND	ND	ND	—	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
4	2+	2+	2+	2+	2+	2+	2+	—	—	2+	2+	—	—	—	2+	2+	—	—	—	—
5	2+	2+	2+	2+	2+	1+	2+	2+	1+	2+	1+	—	—	ND	2+	1+	—	—	—	—
6	2+	2+	2+	1+	ND	ND	ND	—	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
7	2+	—	—	2+	2+	—	2+	—	—	2+	1+	—	—	2+	2+	1+	—	—	—	—
8	—	—	—	2+	—	—	2+	—	—	2+	2+	—	—	—	2+	—	—	2+	—	—
9	2+	—	—	2+	—	—	2+	—	1+	2+	—	—	—	—	2+	1+	—	—	—	—
10	2+	—	—	2+	—	—	2+	—	1+	2+	—	—	—	—	2+	1+	—	—	—	2+
11	—	—	—	1+	2+	—	2+	2+	—	2+	2+	—	—	—	2+	—	—	—	—	—
12	2+	—	—	2+	—	ND	2+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
13	2+	—	—	2+	ND	ND	ND	—	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

ND, not done

Table 5. Immunophenotypic Differences Between T Cell Acute Lymphoblastic Leukemia and T Cell Lymphoblastic Lymphoma

		T Cell ALL	T Cell LL
Reinherz et al[3] stage I	Leu-$2^-3^-6^-(T8^-T4^-T6^-)$	5	5
Reinherz et al stage II	Leu-$2^-3^-6^+(T8^-T4^-T6^+)$	0	3
	Leu-$2^+3^+6^-(T8^+T4^+T6^-)$	0	2
	Leu-$2^+3^+6^+(T8^+T4^+T6^+)$	2	8
	Leu-$2^-3^+6^+(T8^-T4^+T6^+)$	0	1
Reinherz et al stage III	Leu-$2^-3^+6^-(T8^-T4^+T6^-)$	2	1
		9	20

Only cases with complete T cell immunophenotypes (Leu-1 through Leu-6, Leu-9) available included in this table.

phenotype between T cell LL and peripheral T cell lymphomas and mycosis fungoides as seen in Table 6.[15,16] T cell LL is much more likely to show Leu $2^-3^-(T8^-T4^-)$ or Leu $2^+3^+(T8^+T4^+)$ phenotypes, found to be immature thymic phenotypes by Reinherz et al.[3] It is also the only T cell neoplasm expressing Leu-6 (T6) found in the stage II common thymocyte of Reinherz et al. This is keeping with the view that T cell LL represents a neoplastic proliferation of T cells arrested at an immature thymic stage of development. LLs show a greater tendency toward retention of pan-T cell antigens than do peripheral T cell lymphomas. In the current study, Leu-1 staining identified all definite T cell LLs, while Leu-4 and Leu-9 each identified all except one. Leu-1, however, failed to identify two T cell ALLs and has also been found in one non-T cell ALLs (data not shown). Reliance on Leu-5, the sheep erythrocyte receptor, to identify all T cell cases is probably inadequate, as 15% of the LLs and almost half of the T-ALLs were Leu-5 negative.

A potential major discrepancy exists between the current study and previous series of T cell lymphoblastic malignancies concerning Leu-4 expression. Although almost uniformly found in our series, Leu-4 (or T3) expression has been seen in less than half the reported cases in previous large series.[3-5,7] This is undoubtedly due to the method of detection. The previous series have relied on cell suspension studies, which can only detect surface antigen, while the tissue section method of the current study also detects cytoplasmic antigen. A recent comparative study including two of us has shown that there is a discordance between surface and cytoplasmic expression of Leu-4 in lymphoblastic malignancies.[17]

Table 6. Immunophenotypic Differences Between T Cell Lymphoblastic Lymphona, T Cell Lymphoma, and Mycosis Fungoides in Skin[15,16]

	T Cell LL (%) (N = 21)	Peripheral T Cell Lymphoma (%) (N = 50)	Mycosis Fungoides in Skin (%) (N = 25)
Leu-1 expression	100	54	88
Leu-4 expression	95	74	100
Leu-5 expression	85	76	100
Leu-6 expression	60	0	0
Leu-9 expression	95	54	68
Leu-2^-3^- phenotype	43	16	0
Leu-2^-3^+ phenotype	10	64	100
Leu-2^+3^- phenotype	0	12	0
Leu-2^+3^+ phenotype	48	8	0
Ia expression	19	72	12

In our study, most of the T cell LLs could be designated as stage II common thymocyte, according to the scheme of Reinherz et al[3]: three Leu $2^-3^-6^+$ $(T8^-T4^-T6^+)$, two Leu $2^+3^+6^-$), eight Leu $2^+3^+6^+$ $(T8^+T4^+T6^+)$, and one Leu $2^-3^+6^+$ $(T8^-T4^+T6^+)$. This is similar to most previous series[3-5,9] but in contrast to the series of Cossman et al,[7] who found five of eight T cell LLs of helper or suppressor phenotype and no cases lacking both OKT4 (Leu-3) or OKT8 (Leu-2). We have no explanation for this discrepancy, as Leu-2 and Leu-3 expression is consistent from cytoplasm to cell surface.[17] The majority of our T cell ALLs could be designated as intrathymic stage I (five Leu $2^-3^-6^-$) similar to previous series.[4,9] Both the intrathymic stage I T cell ALLs and LLs showed Leu-1 (T1) expression. This is in contrast to Reinherz's scheme of intrathymic differentiation.[3] No data concerning T1 was given when the original scheme was proposed.

This study, in agreement with Ritz et al.,[18] confirms that T cell LLs and ALL may express CALLA, and confirms that T cell lymphoblastic malignancies may also express Ia antigens,[7] although Ia expression has rarely been observed in several cell suspension studies.[9,17] Ia antigens have previously been found on activated T cells as well as on a majority of peripheral T cell lymphomas and a minority of cases of mycosis fungoides involving skin.[15,16] Our study also shows, in agreement with the cases of Aisenberg and Wilkes,[19] that T9 and T10 are not restricted to T cell neoplasms. In fact, OKT9 has been shown to recognize the human receptor for transferrin.[20]

Two cases (cases 22 and 23), both LLs, showed a more unusual immunophenotype suggesting a relationship to both B and T lineage. Both cases failed to express Leu-1 through Leu-5 yet expressed the intrathymic T antigen Leu-6. One of the two cases also expressed Leu-9. In addition, both cases stained weakly for B1 and strongly for B2, although staining for mu was absent. Both cases also expressed Ia antigens, CALLA, T9, and T10. To our knowledge, this phenotype has not been previously described, possibly since previous studies have relied on cell surface detection systems, while the current study using frozen tissue sections also detects cytoplasmic antigen. The cases were confirmed to be lymphoblastic malignancies with the demonstration of terminal deoxynucleotidyl transferase (data not shown). Leu-6 has been previously described on cortical thymocytes, Langerhans cells, and the cells of histiocytosis X.[21,22] Among lymphoid malignancies, Leu-9 has been found to be specific for T lineage.[9] B1 has been reported to be detectable on normal and neoplastic B cells up to the plasma cell stage, and common ALL, but absent on normal T cells and T cell neoplasms.[23] B2 shows a more restricted B cell distribution than does B1 but again is said to be absent on normal or neoplastic T cells.[24] Clinically, case 22, the case with Leu-9 expression, was associated with a mediastinal mass, suggesting a closer relationship to a T cell neoplasm. More sophisticated studies, eg, studies for immunoglobulin or T cell receptor gene rearrangements, may further clarify these cases.

This study confirms the utility of L3B12 as a panhematolymphoid marker to differentiate LL from other small round cell tumors in childhood.[25] L3B12 was reactive in 32 of 33 lymphoblastic malignancies tested in the present study. A wide range in positivity was found with antibodies to the Ki-67 antigen.[26] This antigen has been found to correlate with the proliferative rate of cells. It may potentially be able to discriminate cases that may be more or less responsive to chemotherapy and it may be of interest to determine prospectively whether the expression of this antigen has any prognostic significance.

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Cell-Mediated Amegakaryocytic Thrombocytopenia Associated With Systemic Lupus Erythematosus

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We studied a patient with a rare complication of amegakaryocytic thrombocytopenia (AMT) associated with systemic lupus erythematosus (SLE). To investigate the underlying pathogenesis of AMT, the effects of peripheral blood T cells and serum on human megakaryocyte progenitor cells were studied using in vitro coculture techniques. Mononuclear bone marrow cells (2×10^5) from normal donors produced 33.6 ± 8.8 ($n = 10$) colony-forming unit-megakaryocytes (CFU-M) in our plasma clot system. When 2×10^5 of the patient's T cells were added to the culture system, the number of CFU-M decreased to only 3.5 ± 0.6/2×10^5 bone marrow cells. No evidence of inhibitory effects was found by the addition of the patient's serum and complement to the culture system. The T cells stored at −80 °C on admission were also capable of suppressing autologous CFU-M after recovery from AMT. These results indicate that in vitro suppression of CFU-M from allogenic and autologous bone marrow cells by this patient's T cells provides an explanation for the pathogenesis of AMT associated with SLE.

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A VARIETY OF hematologic abnormalities has been reported in patients with systemic lupus erythematosus (SLE).[1,2] In SLE patients with thrombocytopenia, the number of bone marrow megakaryocytes, as estimated from bone marrow smears, is frequently increased.[3,4] However, thrombocytopenia associated with decreased megakaryocyte is uncommon in SLE.[5] We studied a patient with amegarkaryocytic thrombocytopenia (AMT) associated with SLE. In this study, we attempted to investigate the underlying pathogenesis of AMT associated with SLE using an in vitro clonal assay of human CFU-M and in vitro coculture techniques.

MATERIALS AND METHODS

Patient

A 47-year-old female (S.O.) was diagnosed as having SLE in 1979 on the basis of fever, arthralgia, malar erythema, alopecia, positive antinuclear antibody, and anti-ds DNA antibody. She had been initially treated with 30 mg of prednisolone and continued to make good progress on a lesser amount until three months before admission, when she developed gingival bleeding and ecchymoses. She was found to have thrombocytopenia of 10,000/μL. She was hospitalized elsewhere and then transferred to Tsukuba University Hospital in November 1981 for evaluation of thrombocytopenia. She has no history of blood transfusion. Physical examination on admission revealed numerous petechiae and ecchymoses on both legs. Blood cell count revealed the following: RBC, 3.78×10^{12}/L, Hb, 12.0 g/dL; Hct, 38%, reticulocytes, 0.8%; platelet count, 8×10^9/L; WBC, 8×10^9/L with normal differentiation. The ratio of Tμ to Tγ in T cell fraction was 28.5%:63.5%. Bone marrow aspiration and biopsy showed no megakaryocytes. She was treated with 60 mg of prednisolone daily and a pulse therapy (methylprednisolone 1 g intravenously for three days). The platelet count was elevated to the normal range for three weeks after treatment. Furthermore, the ratio of Tμ to Tγ changed to 53.4%:24.5%. The bone marrow aspirate at this time showed normal cellularity with many mature megakaryocytes. Details of serological tests and clinical course have been previously reported.[6]

Control Subjects

Ten normal subjects, five SLE cases with normal platelet counts (SLE group A), and five SLE cases with thrombocytopenia (less than 50×10^9/L) and increased megakaryocytes (SLE group B) were used as a control. All SLE cases were studied before treatment. Informed consent was obtained from prospective marrow donors before collection of blood specimens and aspiration of bone marrows required for this study. The research procedures and informed consents for this study were approved by the institutional research review committee

Preparation and Freezing of T Cells

Peripheral blood mononuclear cells were separated from freshly drawn heparinized blood by Ficoll-Hypaque density sedimentation[7] from the patient (S.O.), ten cases of SLE, and ten normal donors A T cell-rich fraction was obtained by E rosette centrifugation through Ficoll Hypaque, and the T cells from the patient (S.O) were frozen using a programmed freezing apparatus (Cryo-Med, Mount Clements, Mich). Details of the procedure were previously described.[8] Frozen T cells were stored at −80 °C in a concentration of 2×10^6/mL in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 15% fetal calf serum (FCS; Flow Laboratories, Rockland, Md) and 10% dimethyl sulfoxide (DMSO; Merck, Darmstadt, FRG). When further experiments were required, the T cells were thawed and washed twice by RPMI 1640 supplemented with 2% FCS and then checked for viability with the trypan blue dye exclusion test.

T Cell subsets

T cells bearing receptors for the Fc portion of IgG and IgM were detected by rosette formation with ox red blood cells,[9] which were coated with purified rabbit IgG and IgM antiox red blood cells.

Plasma Clot Megakaryocyte Cultures

Bone marrow cell preparation Bone marrow mononuclear cells were obtained by density centrifugation with Ficoll-Hypaque. The interface mononuclear cell layer was collected and washed twice by α-medium (Flow) supplemented with 2% FCS. Bone marrow cells were finally resuspended in α-medium supplemented with 10% FCS at a concentration of 5×10^6/mL.

Preparation of phytohemagglutinin-leukocyte conditioned medium (PHA-LCM) Mononuclear cells derived from peripheral blood of the normal subjects were cultured in RPMI 1640 supplemented with 10% FCS and 1% PHA (GIBCO) at a concentration of

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2×10^6/mL. The supernatant was harvested after four days of incubation and stored at −20 °C as PHA-LCM.

Culture system. The following materials were used for the plasma clot system: Bovine serum albumin solution (BSA): bovine serum albumin fraction V (Sigma Chemical Co, St Louis) was used. An 8% (wt/vol) BSA stock solution was prepared as described by McLeod et al.[10] L-Asparagine solution: L-Asparagine (Sigma) was dissolved in α-medium at a concentration of 2 mg/mL, filtered through a 0.45-μm filter, and stored at −20 °C until used. This stock solution was diluted 1.10 with α-medium before being added to the culture system. Bovine embryo extract: Bovine embryo extract (BEE) solution (Flow) was diluted 1:5 by α-medium, divided into 2-mL amounts, and stored at −20 °C until used. Erythropoietin (Epo) (Step-III, Connaught Laboratories, Willowdale, Ontario) was diluted to 20 U/mL by α-medium. Supplemented Eagle's minimum essential medium with Hanks' balanced salt solution (supplemented HMEM): The composition of supplemented HMEM was Dulbecco's modified Eagle's medium (Flow), 0.086 g; Hanks' balanced salts without sodium bicarbonate (Flow), 0.99 g; sodium pyruvate (Flow), 0.011 g; 1 mL of MEM nonessential amino acids (GIBCO, 10 mmol/L, 100× concentrate); 1 mL of L-glutamine (GIBCO, 200 mmol/L, 100× concentrate); 0.89 mL of 7% (wt/vol) $NaHCO_3$ solution; 10 mL of heat-inactiviated human type AB serum and 88 mL of double distilled water. Complement: Rabbit lyophilized serum (Cappel Laboratories, Cochranville, Pa) was diluted by α-medium and used as a source of complement.

The plasma clot system was as follows: The mononuclear bone marrow cell suspensions (5×10^5 cells) were mixed with 0.1 mL of BSA, 0.1 mL of BEE, 0.1 mL of L-asparagine, 0.1 mL of PHA-LCM, 0.1 mL of Epo, 0.25 mL of α-medium, 0.05 mL of heat-inactivated human Type AB serum, and 0.1 mL of fresh human type AB citrated plasma. The mixture (0.4 mL) was clotted in a 35-mm culture dish, and 2 mL of supplemented HMEH was placed around the clot as a surrounding medium. The culture dishes were kept for 14 days at 37 °C in a 100% humidified atmosphere of 5% CO_2 in air. The clots were fixed, in situ with 100% methanol for 15 minutes and washed with phosphate-buffered saline (0.01 mol/L, pH 7.2). They were then dried in cool air and stored frozen at −20 °C until immunofluorescence staining.

Identification of megakaryocytic colonies. Immunofluorescent identification using an antiplatelet glycoprotein antiserum was by the method described previously.[11] Briefly, human platelet glycoproteins (HPG) were purified by lithium diiodosalicylate-phenol extraction of human platelets by the method described by Marchesi and Chasis.[12] To prepare antiserum against HPG, New Zealand white rabbits were immunized with a subcutaneous injection of 1 mg of HPG with complete Freund's adjuvant and then intramuscularly injected with 1 mg of HPG every week for four weeks. Blood was collected by ear vein puncture one week after the final injection. Serum was separated and stored at −20 °C. Normal bone marrow smears and frozen sections of lymph node, liver, and spleen were stained to check the specificity of anti-HPG. Megakaryocytes and platelets were intensively fluorescent by this antiserum, although macrophages and eosinophils were slightly stained. Indirect immunofluorescence staining procedure was followed by the method of Hoffman et al.[13] A megakaryocytic colony was defined as an aggregate of more than four fluorescent cells.

CFU-C and CFU-E Assay

Colony-forming unit in culture (CFU-C) assay was carried out using the method of Pike and Robinson.[14] Triplicate cultures were prepared for each marrow. Cultures were maintained at 37 °C in humidified air with 5% CO_2 for 14 days. Cell aggregates of more than 40 cells were called colonies. Colony-forming unit erythroid (CFU-E) assay was carried out using the plasma clot method described by Tepperman et al[15] in the presence of 2 units of erythropoietin (sheep erythropoietin step III; Connaught). Cultures were maintained at 37 °C in humidified air with 5% CO_2 for seven days. Cell aggregates of more than eight benzidin-positive cells were counted as a colony.

Coculture Studies

Normal bone marrow cells from ABO compatible donors were used for all coculture studies. T cells (2×10^5) from the patient (S.O.), ten SLE cases, and ten normal subjects were cocultured with 2×10^5 normal allogenic or autologous bone marrow cells to assay CFU-M. α-Medium was substituted when the T cell suspension was added to the culture system. To study humoral inhibitors, 0.1 mL of serum from the patient, normal donors, and ten SLE patients, and 0.1 mL of complement were added to the culture system. After the recovery of megakaryopoiesis, 2×10^5 of the patient's T cells (frozen on admission) were cocultured with 2×10^5 autologous bone marrow cells. For the coculture studies of CFU-C and CFU-E, 2×10^5 T cells obtained from the patient before treatment and from ten normal subjects were cocultured with 2×10^5 normal allogenic or autologous bone marrow cells to assay CFU-C and CFU-E.

RESULTS

Table 1 shows the control studies from five SLE patients with normal platelet counts (SLE group A), five SLE patients with thrombocytopenia and increased megakaryocytes (SLE group B), and ten normal subjects. Mononuclear bone marrow cells (2×10^5) from ten normal donors produced a mean of 33.6 ± 8.8 megakaryocytic colonies by culturing in the presence of PHA-LCM and Epo. Only 2.6 ± 1.3 megakaryocytic colonies were cloned per 2×10^5 cells in the absence of PHA-LCM and Epo. The mean of megakaryocytic colonies from five cases of SLE group A was 35.5 ± $7.6/2 \times 10^5$ cells. However, the mean of megakaryocytic colonies from five cases of SLE group B was significantly increased to 46.8 ± $11.8/2 \times 10^5$ cells ($P < .05$, as compared by Student's t test). The addition of 2×10^5 T cells from normal donors, five cases with SLE group A, and five cases with SLE group B caused a significant increase in megakaryocytic colony formation from allogenic normal bone marrows to 63.5 ± 10.3, 59.3 ± 11.4, and 60.7 ± $9.5/2 \times 10^5$ cells, respectively ($P < .001$). Also, an addition of autologous T cells caused a similar increase of megakaryocytic colonies in SLE groups A and B as well as in normal subjects. Furthermore, the number of megakaryocytic colonies from normal subjects, SLE group A, and SLE group B were not influenced by the addition of serum and complement.

Table 2 summarizes the effects of T cells and serum from the patient on megakaryocytic colony formation. Only 1.5 ± 0.4 megakaryocytic colonies were cloned per 2×10^5 mononuclear bone marrow cells from the patient. The coculture between 2×10^5 of the patient's T cells and 2×10^5 mononuclear bone marrow cells from a normal donor caused a marked decrease of megakaryocytic colonies to 3.5 ± $0.6/2 \times 10^5$ cells.

When the patient recovered from AMT by combination therapy with a high dose of prednisolone and pulse therapy of methylprednisolone, 2×10^5 of the patient's mononuclear bone marrow cells produced 40.3 ± 8.3 megakaryocytic

Table 1. Effects of T Cells and Serum From Normal Subjects, SLE Group A, and SLE Group B on Megakaryocytic Colony Formation

	Coculture Studies		
	Addition to Culture	Source of Bone Marrow	No of CFU-M/2 × 10^5 Cells
Normal subjects (n = 10)	None	Own	33.6 ± 8.8
	2 × 10^5 T cells	Allogenic normal	63.5 ± 10.3
	2 × 10^5 T cells	Autologous	62.8 ± 11.6
	0.1 mL of serum and complement	Allogenic normal	31.7 ± 6.2
	0.1 mL of serum and complement	Autologous	32.3 ± 6.6
SLE group A (n = 5)	None	Own	35.5 ± 7.6
	2 × 10^5 T cells	Allogenic normal	59.3 ± 11.4
	2 × 10^5 T cells	Autologous	61.4 ± 9.8
	0.1 mL of serum and complement	Allogenic normal	35 7 ± 7.1
	0.1 mL of serum and complement	Autologous	36.0 ± 8.2
SLE group B (n = 5)	None	Own	46.8 ± 11.8
	2 × 10^5 T cells	Allogenic normal	60 7 ± 9.5
	2 × 10^5 T cells	Autologous	75.6 ± 8.3
	0.1 mL of serum and complement	Allogenic normal	40.7 ± 8.5
	0.1 mL of serum and complement	Autologous	45.7 ± 10.4

T cells (2 × 10^5) from normal subjects, SLE group A, and SLE group B were cocultured with 2 × 10^5 autologous and allogenic normal bone marrows. Further, 0.1 mL of serum from normal subjects, SLE group A, and SLE group B were added to culture system with 0.1 mL of complement (rabbit lyophilized serum diluted by α-medium). Values for normal subjects were constructed from ten normal subjects by separate experiments (mean ± SD, n = 10) Values for SLE group A and SLE group B were constructed from five SLE group A and SLE group B by separate experiments (mean ± SD, n = 5). All allogenic normal bone marrows were obtained from different donors.

Table 2. Effects of T Cells and Serum From Patient With AMT Associated With SLE on Megakaryocylic Colony Formation

	Coculture Studies		
	Addition to Culture	Source of Bone Marrow	No of CFU-M/2 × 10^5 Cells
During AMT	None	Own during AMT	1.5 ± 0.4
	2 × 10^5 T cells during AMT	Allogenic normal	3.5 ± 0.6
	0.1 mL of serum during AMT and complement	Allogenic normal	28.6 ± 5 5
After recovery from AMT	None	Own after recovery from AMT	40.3 ± 8 3
	2 × 10^5 T cells frozen during AMT	Own after recovery from AMT	2.7 ± 0.3
	2 × 10^5 T cells after recovery from AMT	Own after recovery from AMT	60 5 ± 7.8
	2 × 10^5 T cells after recovery from AMT	Allogenic normal	65 1 ± 8 4
	0.1 mL of serum during AMT and complement	Own after recovery from AMT	39.6 ± 10.3
	0 1 mL of serum after recovery from AMT and complement	Own after recovery from AMT	41.5 ± 11.5

Each value represents mean ± SD (n = 5). Rabbit lyophilized serum (0.1 mL) diluted by α-medium was used as a source of complement AMT, amegakaryocytic thrombocytopenia.

colonies. In order to study the effect of the patient's T cells on autologous bone marrow cells, 2 × 10^5 of the patient's T cells (frozen on admission) were cocultured with 2 × 10^5 autologous mononuclear bone marrow cells after the recovery of megakaryopoiesis. The number of megakaryocytic colonies was suppressed to 2.7 ± 0.3/2 × 10^5 cells. However, 2 × 10^5 T cells after recovery from AMT failed to suppress the megakaryocytic colonies derived from autologous and allogenic bone marrow cells.

To study the humoral inhibitors influencing megakaryocytic colony formation, the patient's serum (0.1 mL) and complement were added to the culture system. The numbers of megakaryocytic colonies from the patient, normal donors, SLE group A, and SLE group B were not suppressed by the addition of serum and complement, suggesting no evidence of inhibitor in the serum. These coculture studies indicated that T cells were responsible for severe megakaryocytopenia associated with SLE.

In addition, to determine whether T cells from the patient exhibited suppressive effects on autologous and allogenic CFU-C or CFU-E growth or not, 2 × 10^5 T cells from the patient were cocultured with 2 × 10^5 mononuclear bone marrow cells from autologous or allogenic bone marrows. Mononuclear bone marrow cells (2 × 10^5) from normal subjects yielded 105.4 ± 14.5 (mean ± SD, n = 5) of CFU-C and 185.6 ± 21.5 (mean ± SD, n =5) of CFU-E. Similarly, 2 × 10^5 mononuclear bone marrow cells from the patient during AMT yielded 125.4 ± 15.2 of CFU-C and 198.3 ± 21.9 of CFU-E. When T cells obtained from the patient before treatment were added, the mean values of CFU-C and CFU-E were increased to 136.6 ± 18.5 and 245.3 ± 47.6/2 × 10^5 cells, respectively, indicating no suppression of autologous CFU-E and CFU-C growth. T cells from normal subjects also enhanced colony formation of CFU-C and CFU-E (data not shown).

DISCUSSION

Much work has been done on the immune suppression of hematopoiesis in SLE, characterized by major alteration of both the humoral and the cellular branches of immunity.[16] In a previous study, Duckham et al[17] reported that a factor in the serum from patients with SLE inhibited granulocytic colony formation in an in vitro agar system. Also, Fitchen et al[18] described one patient having SLE and IgG inhibitor of granulopoiesis and erythropoiesis. However, in a recent report, the peripheral T cells from SLE patients suppressed the CFU-C growth from allogenic normal bone marrow.[19] These studies indicate that cell-mediated suppression, as well as serum inhibitor, could be responsible for immune cytopenia in SLE.

Acquired AMT is an uncommon disorder that does not represent a single clinical entity.[20] It has been reported in conjunction with SLE without determination of an underlying pathogenesis.[5] It was suggested by Hoffman et al[21] that acquired AMT could involve several pathogenetic mechanisms, including (a) failure of terminal megakaryocytic differentiation; (b) an intrinsic cell defect; (c) abnormal interaction between CFU-M and bone marrow regulatory cells; (d) decreased production of a hematopoietic growth factor; and (e) immunologic attachment on megakaryocytic progenitor cells. They also demonstrated, from their analyses of six cases, that acquired AMT could be due to at least one of two alternative mechanisms: either an intrinsic defect at the level of CFU-M or a circulating cytotoxic autoantibody directed against the CFU-M.[21] More recently, Hoffman et al[22] reported an unusual case of chronic idiopathic thrombocytopenic purpura with amegakaryocytosis due to a humoral antibody cytotoxic to megakaryocyte progenitor cells.

Our control studies showed that the number of CFU-M in SLE group B was significantly higher than those in SLE group A and normal controls, suggesting that the regulatory response to the thrombocytopenic state is not impaired in SLE group B. Also, we failed to suppress the number of CFU-M by the addition of complement and serum from patients in SLE group B as well as from those in group A, indicating no inhibitory humoral antibody to megakaryocyte progenitor cells. These findings suggest that a humoral antibody to megakaryocyte progenitor cells is uncommon in SLE.

T cells may play an important role in the regulation of megakaryopoiesis at the progenitor cell level, although it is well known that T cells stimulate CFU-E and CFU-C growth. The present study indicates that T cells from normal donors, SLE group A, and SLE group B are capable of enhancing the number of CFU-M from allogenic and autologous bone marrow. We have assumed that T cells may secrete a stimulating factor, like a colony-stimulating factor for megakaryocytes (CSF-Meg). However, we could not find a marked difference in T cells from the three groups for enhancing of CFU-M from allogenic or autologous bone marrow.

In contrast, the CFU-M were strongly suppressed when T cells from this patient were cocultured with normal allogenic bone marrow cells. Also, the patient's T cells (frozen on admission) suppressed CFU-M of autologous bone marrow cells after the recovery of megakaryopoiesis. This is a direct demonstration that the patient's T cells are capable of suppressing autologous hematopoietic stem cells. These results indicate that abnormal cell-to-cell interaction between the patient's T cells and megakaryocytic progenitor cells may be responsible for the underlying mechanism of megakaryocytic colony suppression in vitro.

It has recently become possible to quantify subsets of T cells as helper T cells ($T\mu$) and suppressor T cells ($T\gamma$). However, this analysis of subsets of T cells in SLE remains controversial. Morimoto et al[23] reported a selective decrease in $OKT8^+$ cells (suppressor T cells), whereas Bakke et al[24] demonstrated that one half of 28 SLE patients had markedly depressed percentage and absolute number of $OKT4^+$ cells (helper T cells). The patient in the present study showed a decreased number of $T\mu$ cells and a relatively increased number of $T\gamma$ cells. Recently, Worman et al[25] reported an excess of suppressor T lymphocytes in association with AMT in a case of AML. In the present case, an imbalance of T cell subsets may cause a megakaryocytopenia associated with SLE.

This patient developed AMT during an inactive serologic state of SLE. Cline and Golde[16] described a patient having SLE and an IgG inhibitor of granulopoiesis and erythropoiesis and stated that such inhibitors appear to be relatively infrequent in patients with active disease. However, AMT in the present patient responded to a high dose of prednisolone and pulse therapy of methylprednisolone. The analysis of T cell subsets after the recovery of megakaryopoiesis revealed 53.4% of $T\mu$ and 24.5% of $T\gamma$, suggesting the decrease of $T\gamma$ cells to be due to combination therapy.

We conclude that T cells from this patient are capable of in vitro suppression of megakaryopoiesis. For some cases, this may explain the association of AMT with SLE.

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Clinical and Hematologic Characteristics in Acute Leukemia With 11q23 Translocations

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We studied the clinical, morphological, and immunologic characteristics of 11 patients with 11q translocation-associated acute leukemia. There were three patients with t(9;11)(p22;q23), one with a variant of the t(9;11), three with t(11;19)(q23;p13), two with t(1;11)(p32;q23), one with t(10;11)(p15;q22or23), and one with t(11;17)(q23;q25). The breakpoints in chromosome 11 clustered in band q23. The morphological feature was FAB-M5 in two patients, FAB-M2 in one, FAB-L1 in six, and lymphoblastic lymphoma in one. The remaining patient underwent morphological changes from FAB-L1 seen at the time of diagnosis to M5b at relapse. Immunologic marker studies in ten patients revealed that one had T cell type; another pre-B cell type; three CALLA$^-$ Ia$^-$ non-T, non-B type; two CALLA$^-$ Ia$^+$ non-T, non-B type; two monocytic type (positive Fc-receptor); and the remaining one underwent phenotypic changes from CALLA$^+$ Ia$^+$ non-T, non-B type to monocytic type. The patients were usually young; five were under 1 year and two were 9 and 13 years. Hyperleukocytosis was observed in eight of the ten patients with acute leukemia, and two of the eight died of intracranial hemorrhage within two days of admission, associated with disseminated intravascular coagulation. These findings indicate that leukemia with the 11q23 translocation share certain characteristics in common, irrespective of the recipient chromosome, even though the latter may have some influence on the morphological and immunologic phenotype. Our data provide a hypothesis that multipotent stem cells are involved in the genesis of the 11q translocation-associated leukemia.

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IT HAS BEEN REPORTED that reciprocal translocations involving the long arm of chromosome 11 (11q22-24) are associated with acute monocytic leukemia (FAB-M5).[1,2] On the other hand, a 4;11 translocation, in which the same region of No. 11 is involved, was reported in 5% of acute lymphoblastic leukemia.[3] Both the 4;11 translocation and other 11q translocations were often reported in childhood, especially infant, leukemia.[4-6] It was puzzling that the leukemic cells with t(4;11) had a lymphocytic phenotype. Parkin et al[4] and Nagasaka et al[5] proposed, with electron microscopic and immunologic studies, that the leukemic cells with t(4;11) may be in early myeloid differentiation. More recently, Stong et al[7] proved that a leukemic cell line with t(4;11) expressed markers of both early lymphoid and early myeloid cells. There have been few reports, however, on the precise characteristics of leukemia associated with any of the other 11q23 translocations.

We studied clinical and hematologic characteristics in 11 acute leukemia patients with the 11q23 translocation, including t(9;11), t(11;19), t(1;11), t(10;11), and t(11;17), and found the young age of the patients, frequent occurrence of hyperleukocytosis, and protean morphological and immunologic phenotypes of the blast cells. Our data suggest that the leukemia with the 11q23 translocation may be a proliferation of a progenitor cell, which could differentiate into both lymphoid and myeloid cells.

MATERIALS AND METHODS

All patients in this study were Japanese and were hospitalized between March 1982 and May 1985; cases 1, 2, 3, 7, 10, and 11 were admitted to the Saitama Cancer Center Hospital (SCCH); case 4, to the Hokkaido Cancer Center Hospital; case 5, to the National Tochigi Hospital; cases 6 and 8, to the Mie University Hospital; and case 9, to the Kurume University Hospital. The detailed clinical and cytogenetic data on cases 5 and 7 are reported elsewhere. Clinical data were recorded for each patient; these included age, sex, white blood cell count (WBC) with the blast percentage, hemoglobin content, platelet count, serum lysozyme, the antileukemic drugs used for induction therapy, and response to induction therapy. The survival of each patient was recorded in days or months on July 31, 1985

Wright-Giemsa–stained bone marrow (BM) and peripheral blood (PB) smears from all leukemia patients obtained before chemotherapy were reviewed by the three hematologists (Y.K., N.M., M.S.) of SCCH. The cytochemical reactions tested included myeloperoxidase, periodic-acid Schiff (PAS), and α-naphthyl butyrate esterase with or without NaF inhibition. The diagnosis of acute leukemia was made on the basis of the FAB classification.[8] Case 7 presented with systemic lymphadenopathy but had no BM involvement of blast cells. A biopsy sample of a lymph node (LN) was obtained, and the diagnosis for this patient was made on the basis of the International Working Formulation.[9]

Samples for chromosome studies were obtained from BM, PB, or LN before chemotherapy in all patients and in case 5 also at the time of relapse. Cells were cultured for 24 hours without stimulation and for 72 hours with the stimulation of phytohemagglutinin (PHA) for the determination of the constitutional karyotype. Chromosomes were analyzed with regular Giemsa stain and with Q- or G-banding methods. Karyotypes were described according to the International System for Human Cytogenetic Nomenclature.[10]

Immunologic markers of malignant cells were evaluated in ten

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patients at the time of diagnosis or at the time of relapse; these included E, EA (IgG-Fc), and EAC rosette formations and surface and cytoplasmic immunoglobulin determinations. The cell surface phenotype was analyzed also with indirect immunofluorescence using monoclonal antibodies; T cell markers were studied with OKT3, OKT4, OKT6, OKT8, OKT10, OKT11 (Ortho Pharmaceutical Corp, Raritan, NJ), and KOR-T49[11] antibodies, and B cell markers, with B1, B2, and B4 (Coulter Immunology, Hialeah, Fla). Common ALL and Ia antigens were detected with J5 and I2 antibodies (Coulter), respectively. Granulocyte or monocyte antigens were studied with OKM1, OKM5 (Ortho), MY4 (Coulter), and MCS-2 antibodies.[12] Terminal transferase was examined with indirect immunofluorescence using TdT assay kit (Muto, Tokyo).

RESULTS

Cytogenetic data. Results of chromosome studies on the 11 patients are summarized in Table 1. Cases 1, 2, and 3 had a 9;11 translocation (Fig 1), and case 1 had an extra No. 8 in addition to the translocation. Case 4 had two translocations; one between No. 1 and No. 18 and the other between No. 9 with a pericentric inversion and No. 11 (Fig 2), the latter being considered to be a variant of the t(9;11).

Cases 5, 6, and 7 had an 11;19 translocation (Fig 3). At the time of relapse, when the leukemic cells of case 5 had undergone morphological changes from lymphoid to monocytoid cells (see Clinical and Hematologic Data), the patient had 16 cells with the same 46,XX,t(11;19) as the initial karyotype and seven cells with t(11;19) and additional abnormalities. Case 6 had a missing X chromosome in addition to t(11;19). Karyotypic analysis of the peripheral lymphocytes obtained during remission and cultured for 72 hours with the stimulation of PHA showed nine cells with 45,X,−X, three with 47,XXX, and five with various structural chromosome changes, probably caused by chemotherapy and radiotherapy. These chromosome findings indicated that case 6 had Turner syndrome of the mosaic type. The missing X chromosome in the leukemic cells was, therefore, considered to be a constitutional change. In case 7, the 11;19 translocation was found only in LN cells but not in BM cells.

Table 1. Chromosome Findings on 11 Acute Leukemia Patients With 11q23 Translocation

Patient No.	Source of Cells	Number of Cells Examined			Karyotype
		Total	Normal Diploid	Abnormal Clonal	
1	BM	50	0	50	47,XX,+8,t(9;11)(p22;q23)
2	BM	14	0	14	46,XY,t(9;11)(p22;q23)
3	BM	14	0	14	46,XX,t(9;11)(p22;q23)
4	BM	11	3	8	46,XY,t(1;18)(p36;p11),inv(9)(p22q21)t(9;11)(p22;q23)
5	PB	16	3	13	46,XX,t(11;19)(q23;p13)
	PB*	23	0	23	46,XX,t(11;19)/related karyotypes
6	BM	16	0	16	45,X,−X,t(11;19)(q23;p13)
7	LN	41	2	39	46,XY,t(11;19)(q23;p13)
8	BM	6	0	6	46,XX,t(1;11)(p32;q23)
9	PB	37	20	17	46,XY,t(1;11)(p32;q23)
10	BM	11	1	10	47,X,−Y,+8,+21,t(10;11)(p15;q22or23)
11	BM	15	0	15	46,XY,t(11;17)(q23;q25)

*Second chromosome analsyis was performed at the time of relapse. BM, bone marrow; PB, peripheral blood; LN, lymph node.

Cases 8 and 9 had a 1;11 translocation with no other abnormalities (Fig 4). Case 10 had ten cells with a 10;11 translocation, a missing Y and other abnormalities, and one cell with 46,XY. Therefore, the missing sex chromosome of this case is considered to be a change having occurred in the leukemic cell. Case 11 had an 11;17 translocation with no other abnormalities.

Clinical and hematologic data. The clinical and hematologic data on the 11 patients are summarized in Table 2. The patients ranged in age from 4 days to 52 years, and five of them were 11 months or less; one with a variant of t(9;11), two of the three with t(11;19), and two of the two with t(1;11). Case 9 was excluded from the hematologic data analysis because no blasts were observed in his PB and BM. The WBC in the remaining ten patients ranged from $4.4 \times 10^9/L$ to $425 \times 10^9/L$, with a median of $219 \times 10^9/L$. The hemoglobin ranged from 6.0 g/dL to 12.8 g/dL, with a median of 7.9 g/dL. The platelet counts ranged from $19 \times 10^9/L$ to $234 \times 10^9/L$, with a median of $54 \times 10^9/L$. Hepatomegaly was observed in six patients, splenomegaly in four, and lymphadenopathy in five.

Morphology and cytochemistry. Morphological and cytochemical data on the 11 patients are also summarized in Table 2. In case 1, the predominant leukemic cells had a round nucleus with lacy chromatin pattern and a small amount of cytoplasm, occasionally with small numbers of azurophilic granules. Nonspecific esterase reaction was positive in only 2% of leukemic cells of this case, but the reaction was completely inhibited by NaF. Peroxidase reaction was positive. The case was classified as acute monocytic leukemia (AMOL), poorly differentiated (FAB-M5a). In case 2, the predominant leukemic cells were promonocytes and atypical monocytes; they had voluminous cytoplasm with occasional azurophilic granules (Fig 5). Nonspecific esterase reaction was strongly positive, but peroxidase reaction was negative. Case 2 was classified as AMOL, differentiated (FAB-M5b). In case 11, the predominant leukemic cells in the bone marrow were myeloblasts and promyelocytes, and less than 10% of the leukemic cells appeared to be monoblasts or promonocytes. There were some mature granulocytes with abnormal lobulation. Most leukemic cells were positive for peroxidase and nonspecific esterase reactions, the latter of which was completely inhibited by NaF. This case was classified as acute myelogenous leukemia (AML)-M2. However, most BM cells of the case examined after chemotherapy were monoblasts, promonocytes, or atypical monocytes. The leukemic cells of cases 3, 4, 6, 8, 9, and 10 showed lymphoid morphology (Figs 6 and 7). Those of cases 4, 6, 8, and 9 were typical lymphoblasts; ie, the blasts were small (about 15 μ) and had a round nucleus with a clumpy chromatin pattern and scanty cytoplasm. Those of cases 3 and 10 were somewhat atypical for acute lymphoblastic leukemia (ALL); the blasts were small (about 15 μ) and had a round or irregularly shaped nucleus with a rough chromatin pattern and scanty cytoplasm with occasional pseudopods. Nonspecific esterase was positive in only 2% of the blasts of case 3; the reaction was completely inhibited by

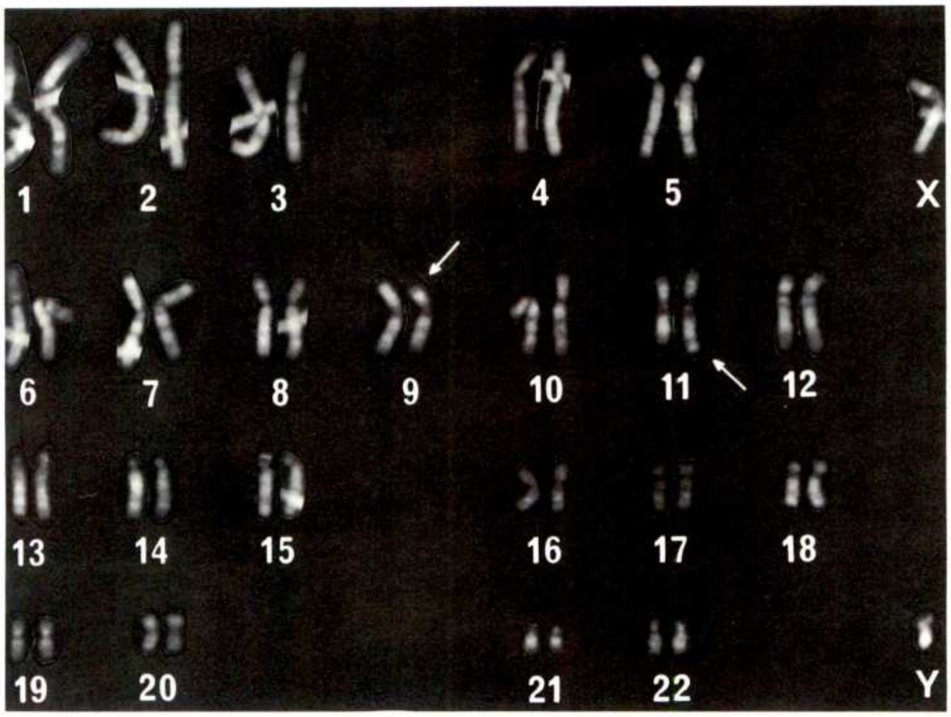

Fig 1. Q-banded karyotype of a cell from case 2. Arrows show abnormal chromosomes forming a 9;11 translocation. The karyotype is 46,XY,t(9;11)(p22;q23).

NaF. Nonspecific esterase was negative in the blasts of case 10. All six cases were negative for peroxidase reaction and were classified as ALL-L1 of FAB type. At the time of diagnosis, leukemic cells of case 5 were small (10 to 12 μ and had a round nucleus with a clumpy chromatin pattern and scanty cytoplasm. The blasts were negative for peroxidase and nonspecific esterase and were classified as ALL-L1 FAB type. The patient entered partial remission but relapsed after two months, when the leukemic cells increased in number (WBC 245 × 10^9/L) with a drastic change in the morpholo-

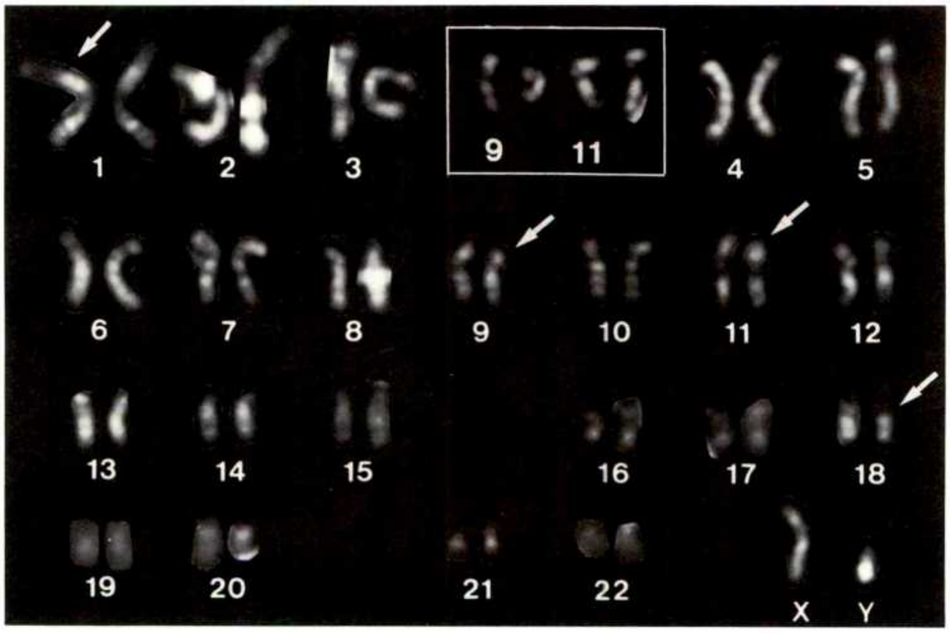

Fig 2. Q-banded karyotype of a cell from case 4. Arrows show two translocations; one between No. 1 and No. 18 and the other between No. 9 with a pericentric inversion and No. 11. Q-banded partial karyotype of another cell is shown in the inset. The karyotype is 46,XY,t(1;18)(p36;p11),inv(9)(p22q21)t(9;11)(p22;q23). inv(9)(p22q21)t(9;11)(p22;q23) is described as t(9;11)(11qter → 11q23::9q21 → 9p22::9q21 → 9qter;11pter → 11q23::9p22 → 9pter) in the detailed system.

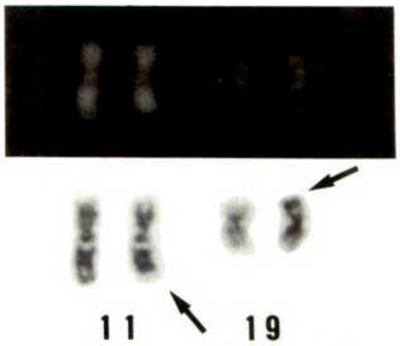

Fig 3. Q- and G-banded partial karyotypes of a cell from case 7. Arrows show abnormal chromosomes forming an 11;19 translocation.

gy. The blasts were larger (about 25 μ) than those observed at the diagnosis and had a nucleus with variable shapes (kidney, lobulated, etc) with a lacy chromatin pattern and large, prominent nucleolei. The cytoplasm was voluminous and bluish gray with fine azurophilic granules. Nonspecific esterase reaction was strongly positive in the cells; the reaction was completely inhibited by NaF. These features were compatible with those of AMOL, differentiated (FAB-M5b).

Histologic examination of the LN of case 7 showed diffuse proliferation of medium-sized round cells with convoluted nuclei. Some of the germinal centers were preserved. The diagnosis of malignant lymphoma, lymphoblastic, convoluted cells was made.[9] Stamp preparation suggested that the blasts could be classified into FAB-L1 type.

Immunologic surface marker studies. Immunologic markers were studied in ten of the 11 patients—in cases 1, 3, 6, 7, 8, 9, 10, and 11 only at the time of diagnosis, in case 4 only at the time of relapse, and in case 5 at both times. The results are summarized in Table 3. The immunologic phenotypes of lymphoid leukemic cells in cases 3, 6, and 10 were essentially the same; they formed no E, EA, and EAC rosettes, bore no sIg, and were nonreactive with various monoclonal antibodies, including J5, I2, anti-T cell, anti-B cell, and anti-granulocyte/monocyte antibodies.

The lymphoid leukemic cells of cases 4, 5 at diagnosis, and case 8 also formed no rosettes and bore no sIg but expressed Ia antigen; in addition, those of case 5 at diagnosis reacted with J5 and MCS-2.

The phenotypes of monocytic leukemia cells of case 1 and those of case 5 at relapse were essentially the same; the cells had Fc receptors, the nonspecific bindings of which obscured the other immunologic markers. The leukemic cells of case 11 also had Fc receptors and reacted with antimonocyte monoclonal antibodies.

The LN cells of case 7 were reactive with some T cell antibodies, including OKT6, OKT8, and OKT10, but were nonreactive with OKT4 and OKT11; these findings suggested that the lymphoblasts were T cells of thymocyte origin. In case 9, surface and cytoplasmic μ-chains were present on 30% and in 90% of the leukemic cells, respectively, and surface and cytoplasmic κ-chains were positive on 30% and in 80% of the leukemic cells, respectively. The cells reacted with B4 and MY4 antibodies. This case was classified as pre-B cell type.

Serum lysozyme. Serum lysozyme value was examined in eight cases—in cases 1, 2, 3, 7, 9, 10, and 11 at the time of diagnosis and in case 5 at the time of diagnosis and at relapse. The value was in the normal range in five cases with lymphoid leukemic cells (cases 3, 5 at diagnosis, 7, 9, and 10) and elevated in four cases with monocytic or granulocytic leukemic cells (cases 1, 2, 5 at relapse, and 11) (Table 2).

Therapy. Cases 1 and 2 with t(9;11) died of intracranial bleeding associated with disseminated intravascular coagula-

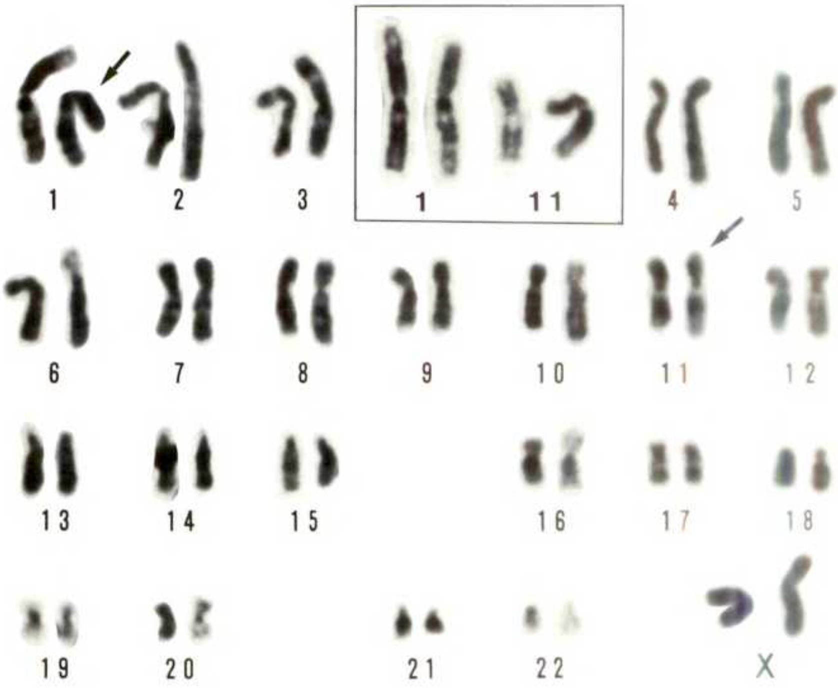

Fig 4. G-banded karyotype of a cell from case 8. Arrows show abnormal chromosomes forming a 1;11 translocation. G-banded partial karyotype from another cell is in the inset. The karyotype is 46,XX,t(1;11)(p32;q23).

Table 2. Clinical and Hematologic Data on 11 Acute Leukemia Patients With 11q23 Translocation

Patient No.	Age(yr)/ Sex	Hgb (g/dL)	Platelets (× 10^9/L)	WBC (× 10^9/L)	Blasts in PB (%)	FAB	Peroxidase	ANBE	NaF Inhibition	PAS	Serum Lysozyme (g/mL)	Therapy	Response to Therapy	Survival
t(9;11)														
1	9F	11.4	73	382.0	98.0	M5a	+	±	+	ND	141.6	DNR,AC	IF	2 d
2	27M	11.1	117	180.0	bl 16.0 prom 27.0 mono 48.0	M5b	−	++	+	ND	221.0	—		1 d
3	22F	7.1	20	4.4		L1	−	±	+	ND	6.8	P,VDS -DNR,AC,	NR-IF	13 d
4	5/12M	8.4	36	354.0	92.0	L1	−	ND		+	ND	V,L,P	CR-Re	14 mon
t(11;19)														
5 D	0/12F	7.3	56	299.4	94.5	L1	−	−		dot+	16.2	V,P-V,P, MTX,Mp	PR-Re	8 mon
R				245.6	93.0	M5b	+	+	+	+	1140.0	DX,BH-AC	CR-Re	
6	11/12F	12.8	106	5.1	99.0	L1	−	ND		−	ND	V,P	CR	23 mon+
7	13M	13.2	164	3.3	0.0	(LBL)	−	ND		ND	10.0	VDS,DX, Cy,P	CR	6 mon+
t(1;11)														
8	8/12F	4.0	28	425.0	97.0	L1	−	ND		−	ND	V,P,Cy, MTX,BLM	CR	19 mon*
9	4/12M	7.1	19	104.0	56.0	L1	−	−		−	2.1	Cy,V,AC	CR	9 mon+
t(10;11)														
10	46M	6.0	234	82.8	95.0	L1	−	−		−	2.1	DX,V,P -DHAD,AC	NR-IF	1 mon
t(11;17)														
11	52M	10.8	51	257.3	82.0	M2	+	++	+	ND	164.0	DNR,AC -DHAD,Et	NR	1 mon+

AC, cytosine arabinoside; BH-AC, enocitabine; BLM, bleomycin; Cy, cyclophosphamide; DHAD, mitoxantrone; DNR, daunorubicin; DX, doxorubicin; Et, etoposide; L, L-asparaginase; Mp, 6-mercaptopurine; MTX, methotrexate; P, prednisolone; V, vincristine; VDS, vindesine; D, at the time of diagnosis; R, at the time of relapse; ND, not done; IF, induction failure; NR, no response; CR, complete remission; PR, partial remission; Re, relapse; mon, months; d, days; LBL, lymphoblastic lymphoma; + after survival indicates that the patient is alive.

*Case 8 died of pneumonia.

tion (DIC) and probably leukostasis in the brain arteries, right after or before the initiation of chemotherapy, respectively. Case 11 presented with DIC-associated subcutaneous bleeding. In this patient, intracranial bleeding was prevented by leukapheresis and cranial irradiation. However, he failed to enter remission by chemotherapy with daunorubicin and cytosine arabinoside and is currently being treated with a second regimen consisting of etoposide and mitoxantrone. The other eight patients received vincristine or vindesine and prednisolone with or without other antileukemic drugs; cases 4, 6, 7, 8, and 9 entered complete remission, among whom case 4 relapsed soon. Case 5 achieved only a short, partial remission, and cases 3 and 10 had no response to the chemotherapy. The chemotherapy regimen of cases 3, 5, and 10 was changed to that with daunorubicin, doxorubicin, and mitoxantrone, respectively, and cytosine arabinoside or enocitabine. Case 5 entered complete remission, but cases 3 and 10 died of sepsis with extreme BM hypoplasia.

DISCUSSION

A review of the literature and our data showed a total of 55 patients with an 11q translocation, including 20 with t(9;11),[6,13-21] one with a variant of t(9;11), 12 with t(11;19),[1,6,22-28] three with t(1;11),[29] nine with t(10;11),[1,6,13,16,30,31] and ten with t(11;17).[1,13,15,18,26,32-35]

The breakpoint in 9p was somewhat different among the

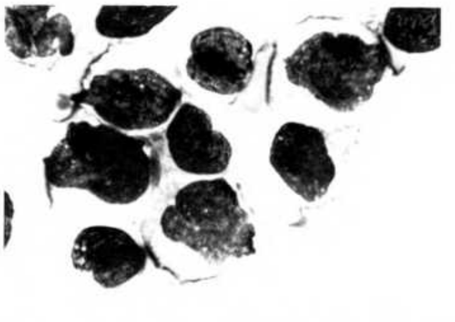

Fig 5. The leukemic cells in the bone marrow aspirate of case 2 consist of promonocytes and atypical monocytes with voluminous cytoplasm. This case was classified as AMOL (FAB-M5b). The leukemic cells had t(9;11).

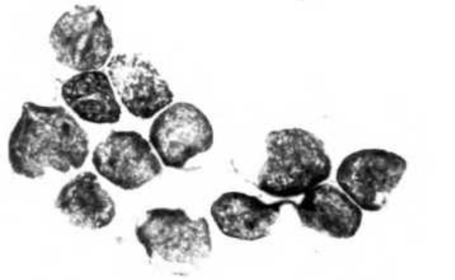

Fig 6. The leukemic cells in the bone marrow aspirate of case 3 are small and have a round nucleus with scanty cytoplasm. The case was classified as ALL-L1 (FAB). The leukemic cells had t(9;11).

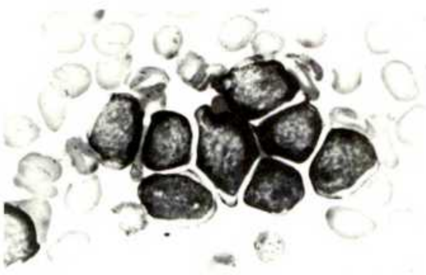

Fig 7. The leukemic cells in the peripheral blood of case 6 are small and have clumpy chromatin pattern and scanty cytoplasm. This case was classified as ALL-L1 (FAB). The leukemic cells had t(11;19).

cases with the t(9;11); in half of them the break was in 9p22 and in the other half it was in 9p21. This discrepancy is apparently related to the banding method used for the analysis.

We interpreted that one of the breakpoints of the t(11;19) in our cases was in the short arm of No. 19. One case in the literature reports a breakpoint in 19q rather than in 19p.[22] Because it is sometimes difficult to judge whether the break of No. 19 is in the short arm or in the long arm, it is possible that t(11;19)(q23;p13) and t(11;19)(q23;q13) may be different interpretations of the same translocation. Two of our cases had t(1;11)(p32;q23), and another in the literature was described as having t(1;11)(p33?;q25).[29] These two 1;11 translocations may also be different interpretations of the same translocation.

The breakpoints in the t(10;11) are diverse; 10p15 in two patients,[13] 10p14 in three,[1,16] 10p13 in one,[1] 10p11 in two,[6,31] and 10p1? in one[30]; 11q13-14 in five[1,16,30] and 11q22-24 in four.[6,13,31] Of the t(11;17), the break in No. 11 occurred in 11q23-24 in nine of the ten cases, but the breakpoint in No. 17 was different among the ten cases reported on; six had the break in 17q25,[13,18,32-34] three in 17q21,[15,26,34] and one in 17q23.[1] It is obvious, therefore, that the breakpoints in the 11q translocation cluster in band q23 except for t(10;11), in which 11q13-14 and 11q23-24 breaks occur in almost equal frequencies. Recently, protooncogene *ets* was assigned to the band 11q23-24.[36]

In the study of our cases with the 11q23 translocation-associated leukemia, we found that morphological phenotypes of the blasts were monocytic in two, myelocytic in one, and lymphocytic in seven. The remaining one underwent morphological changes from lymphoid cells at the time of diagnosis to monocytoid cells at relapse. In the review of the literature, the 11;19 translocation has been found in six cases with acute nonlymphocytic leukemia (ANLL),[1,6,22-24,26] two with ALL,[25,27] and one with lymphoblastic lymphoma,[28] the latter disease being considered a different manifestation of T cell ALL. On the other hand, the 9;11, the 10;11, and the 11;17 translocations have been reported only in patients with ANLL, mostly with a diagnosis of AMOL (FAB M5), with the exception of the two cases with t(9;11) and the one with t(10;11) in our series, which were all associated with ALL. The three cases with t(1;11) in the literature and the present series were classified as ALL. Therefore, the morphological phenotype was more variable in blasts with t(11;19) than in those with t(9;11), t(10;11), t(11;17), or t(1;11).

The immunologic phenotype was also diverse in our patients with the 11q23 translocation. The lymphoid blasts of cases 3, 6, and 10 possessed no immunologic markers and were therefore considered to be totally undifferentiated. The lymphoid blasts of cases 4 and 8 expressed only Ia antigen and were considered to be a little more differentiated than those of the previous three cases. The lymphoid blasts of case 5 at diagnosis may have been in early myeloid differentiation because they reacted with antimyeloid antigen while showing negative peroxidase reaction. The lymphoid blasts of case 9 were pre-B cells, but curiously enough, they reacted with an antimonocyte antibody. Similar lineage infidelity was reported in ANLL and human leukemia cell lines.[7,37,38] The lymphoid blasts of case 7 with t(11;19) were T cells at the thymocyte level. Almost the same immunologic phenotype

Table 3. Immunologic Findings on 10 Acute Leukemia Patients With 11q23 Translocation

Patient No.	E	EA (IgG-Fc)	EAC	sIg	cIg	I2	J5	OKT3	OKT4	OKT6	OKT8	OKT10	OKT11	B1	B2	B4	TdT	MCS2	MY4	OKM1	OKM5
t(9;11)																					
1 D	−	+	−	(+)		(+)	(+)	(+)*										−			
3 D	−	−	−	−		−	−	−*										−			
4 R	−	−		−		+	−							−							
t(11;19)																					
5 D	−	−	−	−		+	+	−*										+			
R	−	+	+	(+)		(+)	(+)	(+)*										(+)			
6 D						−	−	−	−		−	−		−						−	
7 D	−	−		−	−	−	−	−	−	+	+	+	−	−	−	−	−				
t(1;11)																					
8 D				−	−	+	−	−	−		−	−	−	−						−	
9 D				+	+	−	+		−	−	−		−	−	−	+		−	+		
t(10;11)																					
10 D	−	−	−	−		−	−	−				+	−	−	−	−	−		−	−	−
(t11;17)																					
11 D	−	+	+	(+)		−	−					+	−	−			−		+	+	−

Parentheses indicate that nonspecific bindings to Fc receptors obscured the reaction. D, at the time of diagnosis; R, at the time of relapse.

*A pan-T cell antibody, KOR-T49, was used instead of OKT3.

was reported in a child with t(11;19)-associated acute T cell leukemia.[28]

The monocytoid cells of cases 1 and 5 at relapse and the myeloblasts of case 11 had Fc receptors. Monocytoid cells with Fc receptors of case 1 and lymphoid cells with no immunologic markers of case 3 had t(9;11) in common. These findings and refractoriness to the chemotherapy regimen for ALL observed in case 3 suggest that the latter cells may have been in an undifferentiated stage of the same cell lineage as the former cells, and not in the true lymphocytic cell lineage. The lymphoid cells of case 4 with a variant of t(9;11) also showed an immunologic phenotype similar to that of case 3, except for a positive reaction to I2 antibody, which is known to react with some monocytic cells. These cells may also have been in an undifferentiated stage of the monocytic cell lineage.

The leukemic cells of case 5 were lymphocytic at the time of diagnosis but were monocytic when she relapsed after a short, partial remission. The features observed in case 5 are consistent with those in cases with biphenotypic leukemia.[39] The wide spectrum of morphological and immunologic phenotype observed in our cases and biphenotypic features observed in case 5 provide a hypothesis that multipotent stem cells, which are capable of differentiating into both lymphoid and myeloid cells, are involved in the genesis of the 11q23 translocation-associated leukemia.

The patients with leukemia with the 11q23 translocation are usually children or young adults. The median age was 22 years in the 21 patients with t(9;11), including a patient under 1 year of age, 4 years in the 12 patients with t(11;19), including six under 1 year, 6 months in the two patients with t(1;11); no age was described in another with t(1;11), 16 years in the nine patients with t(10;11), including one under 1 year, and 46 years in the nine patients with t(11;17), including two under 1 year; no age was described in another with t(11;17). These data indicate that the 11;19 and 1;11 translocations are especially common in infants.

Hyperleukocytosis may be another characteristic of this type of leukemia. In eight of the ten patients with 11q translocation-associated leukemia, the initial WBC count was greater than 80 × 10^9/L. Patients with the 11q23 translocation, especially t(9;11), and hyperleukocytosis may be at extremely high risk to develop DIC-associated intracranial bleeding.

Nine patients in the series were evaluable for response to chemotherapy. They received various regimens and their responses were different. Remission was achieved in all six children but in none of the three adults. Even though two of the children relapsed soon, our experience suggested that high-risk ALL regimens might be a reasonable choice for childhood ALL with the 11q translocation. On the other hand, adult patients with the 11q translocation had better be treated with a regimen for ANLL regardless of the leukemic cell morphology, although they may show only a limited response to a standard regimen with daunorubicin and cytosine arabinoside.

In conclusion, acute leukemia with the 11q23 translocation may be characterized by the young age of the patients, hyperleukocytosis, and protean morphological and immunologic phenotypes. Occasional biphenotypic features of leukemia cells may be another characteristic. These findings are similar to those of patients with t(4;11).[4,5] Thus all leukemias with the 11q23 translocation may form the same subgroup in acute leukemia, although the recipient chromosomes of the translocation may have some influence on the morphological and other clinicopathologic characteristics. Morphologic, immunologic, and cytogenetic studies on 11q23 translocation-associated leukemia may be useful in extending our knowledge of hemopoietic stem cells and their differentiation.

ACKNOWLEDGMENT

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In Vitro Modulation of Alkaline Phosphatase Activity in Neutrophils From Patients With Chronic Myelogenous Leukemia by Monocyte-Derived Activity

By Tatsuki Matsuo

To clarify the underlying mechanism of low neutrophil alkaline phosphatase (NAP) activity in chronic myelogenous leukemia (CML), CML neutrophils were cultured in liquid medium with different numbers of monocytes. Alkaline phosphatase activity in CML neutrophils, assessed cytochemically, increased with the numbers of monocytes. NAP activity was not induced by the interaction between neutrophils and monocytes, but by the presence of a monocyte-derived soluble activity. NAP activity in normal neutrophils was also lowered by depletion of monocytes from culture medium. Under such monocyte-depleted conditions, both CML and normal neutrophils proliferated and differentiated to produce mature neutrophils. Thus induction of NAP activity can be modified in vitro by changing the amount of NAP-inducing activity released from monocytes. However, whether a reduction of NAP-inducing activity in CML neutrophil is the cause of low NAP activity in vivo remains uncertain.

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NEUTROPHIL ALKALINE PHOSPHATASE (NAP) activity is uniformly low in patients with chronic myelogenous leukemia (CML) at the time of diagnosis[1] and is known to increase during an infectious[2] or inflammatory complication[3] or clinical remission with chemotherapy.[1] Recently, CML neutrophils have been demonstrated to recover NAP activity in vitro[4,5] and in vivo.[6] NAP activity of CML neutrophils also increases after transfusion in febrile patients.[7] Low NAP activity of CML neutrophils in vivo has been considered to be a result of some still unidentified environmental influence.[1,7] In the present study, whether in vitro NAP activity of CML neutrophils could be maintained at a low level under liquid culture conditions was examined.

MATERIALS AND METHODS

Patients. Fifteen untreated patients in the chronic phase of CML were studied. Five patients were male and ten, female. They ranged from 23 to 76 years old (mean, 50.2). The peripheral blood leukocyte count ranged from 11.4 to 259.0 × 10^9/L (mean, 113.4 × 10^9/L). Ph^1 chromosome was detected in 14 patients. Three healthy volunteers were included as controls. Informed consent was obtained from all subjects.

Cell preparations. Bone marrow and peripheral blood were obtained with heparinized syringes. Light-density (LD) cells were separated from each sample by Ficoll-Conray gradient centrifugation (1.077 g/μL).[8] Nonphagocytic (NP) cells were obtained by depleting LD cells of phagocytes using iron-particle phagocytosis.[9] Briefly, LD cells (5 × 10^6) were incubated in 1 mL of α-medium (Flow Laboratories, Irvine, Scotland) with 40 mg/mL carbonyl iron powder (GAF Corp, New York) and 20% fetal bovine serum (FBS; Flow Laboratories, North Ryde, Australia) for 30 minutes at 37 °C. NP cells were recovered after removing iron-phagocytosed cells with a magnet. Both cell samples were washed three times and resuspended in complete medium.

Granulocyte-macrophage colony assay. The granulocyte-macrophage (GM) colony assay was performed according to the method of Iscove et al.[10] A giant cell tumor-conditioned medium (GCT-CM; GIBCO, Grand Island, NY) was used as the exogenous colony-stimulating activity (CSA). LD cells (1 × 10^5) were plated in a plastic Falcon Petri dish (35-mm in diameter) containing 1.0 mL of α-medium with 0.88% methyl cellulose, 10% GCT-CM, and 20% FBS and then incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air for seven days. The dishes were assessed under an inverted microscope for colonies containing 20 or more cells. Fifty individual colonies were aspirated from each sample by a finely drawn out microcapillary tube, and smears of each colony were prepared by a Cytospin (Shandon Southern Products, Cheshire, England).[11]

Liquid culture. Cells (1 × 10^6) in 1 mL of α-medium with 20% FBS were incubated in Falcon plastic Petri dishes (35-mm in diameter) in duplicate for three to seven days at 37 °C in a humidified atmosphere of 5% CO_2. Both adherent and nonadherent cells were completely harvested by a Pasteur pipette after scraping the dishes gently with a rubber policeman. Average volume recovery was 0.9 mL/per dish. Cell viability was at least 90% by trypan blue dye exclusion. Three slides were prepared from each dish by cytocentrifugation and subjected to alkaline phosphatase staining, and May-Grünwald-Giemsa staining for the analysis of cell composition and dual esterase staining[12] for the detection of the number of monocytes and macrophages.

Liquid culture on agar layer. A liquid-on-agar culture was used to investigate the effect of monocyte-derived humoral activity on the induction of NAP activity in NP cells. Cells (1 × 10^6) were plated in the underlayer consisting of 1 mL of 0.65% agar in α-medium with 20% FBS, at various numbers of monocytes by mixing LD cells and NP cells. NP cells (1 × 10^6) in a 1 mL of α-medium with 20% FBS were overlaid. No cells were found in the overlayer during the culture period when only α-medium with FBS was overlaid. After a seven-day incubation, NP cells in the overlayer were gently resuspended and completely harvested with a Pasteur pipette by confirming under an inverted microscope. Cytospin smears of the cells were stained with alkaline phosphatase.

Alkaline phosphatase stain and scoring system for enzyme activity. The naphthol AS-MX phosphate/fast blue RR-staining has high cytochemical sensitivity and reveals sharp localization of enzyme activity.[13] The correlation coefficient between enzyme activities estimated by this method and those by colorimetric assessment was very high ($r = .87$). Smears were fixed for five seconds at −3 °C with 10% formalin in methanol. After washing in running water, slides were mounted with 2 mL of reaction medium and incubated for two hours at 37 °C. The reaction medium was made by adding 1 mg of fast blue RR (Sigma Chemical Company, St Louis) to 1 mL of stock solution, which was prepared by dissolving 10 mg of naphthol AS-MX phosphate (Sigma) in 4 mL of dimethyl formamide and adding 120 mL of water and 76 mL of 0.2 mol/L propanediol buffer. This stock solution was kept refrigerated and was thoroughly mixed before use. After washing in running water, the slides were counterstained with 1% safranine-O for three minutes. NAP activity appeared as blue granules.

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The enzyme activity in each neutrophil was graded according to the number of blue granules in the cytoplasm: type 0 (no granules), type I (less than five granules), type II (less than 30 granules), type III (more than 30 granules, unevenly distributed), type IV (many granules, evenly distributed with small gaps), and type V (numerous granules, densely distributed). The sum of rating in 100 mature neutrophils (restricted to band and segmented forms) was considered as the NAP score for a particular specimen. The normal range of peripheral blood NAP score was from 158 to 295 (n = 20). In the liquid culture experiments, 400 mature neutrophils (band and segmented forms), 200 from each of the duplicated liquid culture dishes were graded and the mean NAP score for 100 cells was calculated in each experiment.

Preparation of LD cell conditioned medium. LD cells (5 × 10^6) containing monocytes were suspended in 1 mL of α-medium with 20% FBS. They were incubated for five days at 37 °C in a humidified atmosphere of 5% CO_2. The supernatant was harvested by centrifugation (3,000 rpm) and filtering with 0.45-μm Millipore filter (Millipore Corp, Bedford, Mass).

Neutrophil count. The absolute number of nucleated cells harvested from liquid cultures was counted using a hemacytometer. Neutrophil numbers were calculated from each differential count on May-Grünwald-Giemsa–stained smear.

Statistics. Student's *t* test used to assess the statistical significance between comparable experimental groups.

RESULTS

NAP activity of granulocyte-macrophage colony cells. There was no colony in which all mature neutrophils lacked NAP activity. The NAP score for cells in 50 colonies in each experiment was always high. There was no significant difference between peripheral blood and bone marrow samples (Fig 1).

NAP activity of CML light-density cells in liquid culture. The NAP score of band and segmented forms of neutrophils in the LD cell preparation was regarded as the preincubation NAP score. The NAP score began to increase on day -3 of culture and reached the maximum between days 5 and 7 (Fig 2A). The maximum NAP score in this culture condition seldom exceeded the normal upper limit. Similar results were obtained from both peripheral blood and bone marrow samples.

NAP activity of CML nonphagocytic cells in liquid culture. The CML NP cells were cultured in liquid medium to avoid the effect of monocytes, which are known to produce various biological substances, including CSF.[14,15] The NAP score of band and segmented forms of neutrophils in the NP cell preparations was regarded as the preincubation NAP score. The NAP activity did not increase during the seven-day incubation period (Fig 2B). Similar results were obtained from both peripheral blood and bone marrow samples.

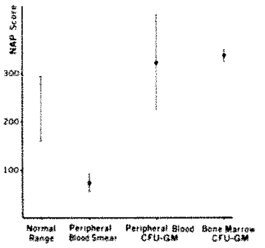

Fig 1. Neutrophil alkaline phosphatase (NAP) score in band and segmented forms of neutrophils derived from granulocyte-macrophage colony (CFU-GM) in five patients with chronic myelogenous leukemia. The vertical line indicates mean ± SD (n = 5).

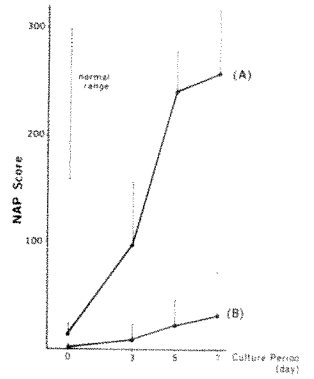

Fig 2. Change in neutrophil alkaline phosphatase (NAP) score in a liquid culture system using light-density cells (A) and nonphagocytic cells (B) depleted of monocytes from the bone marrow of four patients with chronic myelogenous leukemia. The mean ± S.D. (n = 4).

Relationship between the induction of NAP activity and monocyte number. Isolation of monocytes from CML peripheral blood or bone marrow was difficult because of their relative scarcity. Therefore, mixtures of CML LD cells and CML NP cells were incubated in liquid culture at various ratios to ascertain the monocyte number at which NAP activity was induced. The mean percentage of monocytes estimated using dual esterase stain were 1.6%, 0.9%, 0.4%, and 0.2%, when the percentage of LD cells in the mixture was 100%, 50%, 20%, and 0% (all NP cells), respectively. The NAP activity began to increase when the mixture contained 20% LD cells and reached a plateau when the mixture contained 50% LD cells (Fig 3). The results obtained with peripheral blood samples were similar to those obtained with bone marrow samples.

Dependency of NAP activity induction on monocyte-derived soluble activity. To investigate whether the increase of NAP activity in LD cells depended on the

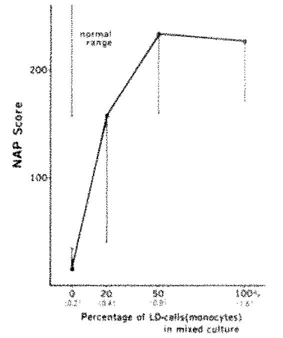

Fig 3. Neutrophil alkaline phosphatase (NAP) score in coculture of light-density cells and nonphagocytic cells obtained from the bone marrow of four patients with chronic myelogenous leukemia at four mixing ratios of monocytes. The mean ± S.D. (n = 4).

interaction between neutrophils and monocytes or on the soluble factor produced by monocytes, NP cells were cultured as target cells in liquid medium over the agar underlayer that contained various mixtures of LD cells and NP cells. The NAP activity of CML NP cells in the upper liquid increased proportionately to the CML LD cell percentage of the agar underlayer (Fig 4).

NAP-inducing activity and CSA in CML LD cell-conditioned medium. Autologous LD cell-conditioned medium was added to the liquid culture of NP cells to induce NAP activity, and to the CFU-GM assay as a CSA. As shown in Fig 5, the LD cell-conditioned medium from case A had strong NAP-inducing activity as well as measurable CSA. The conditioned medium from case B showed weak NAP-inducing activity but no CSA. Neither NAP-inducing activity nor CSA were observed in the conditioned medium from case C.

NAP-inducing activity of exogenous CSA sources. Various amounts of CSA sources were added to the liquid culture of CML NP cells. Preliminary tests disclosed that the human placental conditioned medium (HPCM), GCT-CM, and CSF-Chugai[16] (Chugai Pharmaceutical Co, Tokyo) stimulated normal bone marrows to form a similar number of CFU-GM. CSF-Chugai, highly purified by depleting the inhibitor(s), was diluted to make 100 U/mL at a 10% concentration. These three CSA sources consistently induced NAP activity in a similar dose-dependent manner (Fig 6). The NAP score began to increase at a concentration of 1% and reached the upper limit of the normal range at a concentration of 10%, which is usually used in CFU-GM assay.

Monocyte dependency of NAP activity induction in normal neutrophils. The NAP scores of normal bone marrow LD cells and NP cells from the three control subjects were very low during preincubation. During the seven-day culture, the NAP score increased considerably in the LD cells (Fig 7A) and remained very low in the NP cells (Fig 7B).

Neutrophil growth in liquid culture. Both CML neutrophils and normal bone marrow neutrophils proliferated for seven days in all liquid cultures. In particular, CML neutrophils grew at a significantly higher rate in the presence of LD cells than with NP cells ($P < .01$) (Fig 8), even though there was little difference between their initial maturational composition in the neutrophil compartment (Fig 9). The mean ratio of monocytes-macrophages to neutrophils at days 0, 3, 5, and 7 were 0.015, 0.027, 0.035, and 0.046 in CML LD cells and 0.081, 0.115, 0.112, and 0.120 in normal LD cells, respectively. The mean ratios of monocytes-macrophages to neutrophils in NP cells from both CML and normal bone marrows did not exceed 0.015 until day 5. The ratio at day 7 was 0.024 in CML and 0.013 in normal subjects. The Ph[1] chromosome was detected during the culture period (data not shown).

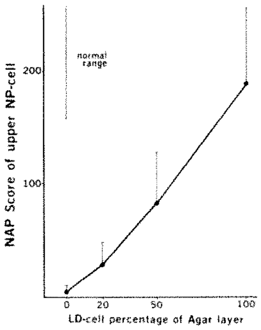

Fig 4. Neutrophil alkaline phosphatase (NAP) score in nonphagocytic (NP) cells cultured for seven days in liquid-over-agar layer with a mixture of NP cells and light-density cells obtained from the bone marrow of three patients with chronic myelogenous leukemia. The mean ± S.D. (n = 3).

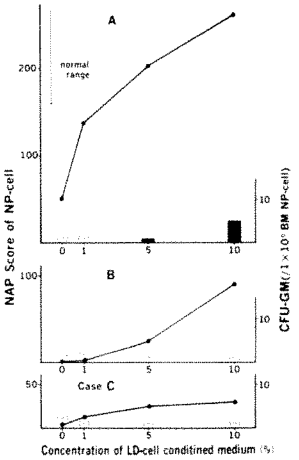

Fig 5. Relationship between neutrophil alkaline phosphatase (NAP)-inducing activity and colony-stimulating activity in autologous light-density (LD) cell-conditioned medium of bone marrow (BM) cells obtained from three patients with chronic myelogenous leukemia. The solid line indicates NAP score and the columns, the number of granulocyte-macrophage colony (CFU-GM).

DISCUSSION

Because GM-colony forming cells from CML patients showed high NAP activity in the presence of a CSA source (GCT-CM), the factor responsible for the consistent elevation of NAP activity in liquid culture of CML LD cells was

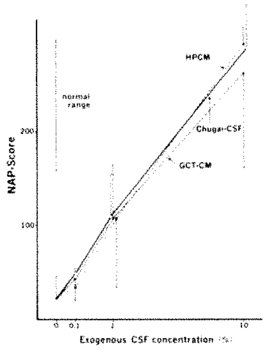

Fig 6. The effect of colony-stimulating activity sources in inducing neutrophil alkaline phosphatase (NAP) activity in nonphagocytic cells obtained from bone marrow of three patients with chronic myelogenous leukemia. A giant cell tumor-conditioned medium (GCT-CM), a human placental conditioned medium (HPCM), and colony-stimulating factor (CSF)-Chugai showed similar results. The mean ± S.D. (n = 3).

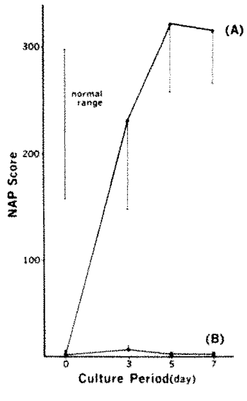

Fig 7. Change in neutrophil alkaline phosphatase (NAP) score in liquid culture system using light-density cells (A) and nonphagocytic cells (B) obtained from the bone marrow of three normal volunteers. The mean ± S.D. (n = 3).

suspected to be colony-stimulating factor (CSF) released from monocytes. The NAP activity of NP cells with less than 0.2% monocytes was not significantly increased after seven days of liquid culture. During this period, NP cells consistently proliferated to produce mature neutrophils, although their growth rate was lower than that of LD cells. Furthermore, modification of monocyte number in CML cells by mixing the LD cells and NP cells at various ratios clearly demonstrated that NAP activity elevates proportionally to the number of monocytes present. NAP activity was also induced in NP cells cultured in the upper liquid with LD cells plated in the under agar layer. The degree of induction was again dependent on the number of monocytes in the underlayer. These observations indicate that NAP activity of CML neutrophils is induced in vitro, not by interaction between neutrophils and monocytes, but by soluble activity released by the monocytes.

The conditioned media of CML LD cells obtained from one of the three patients revealed simultaneous expression of both NAP-inducing activity and CSA, although CSA was relatively weak compared with the considerably high NAP-inducing activity. The three exogenous CSA sources showed similarly high NAP-inducing activity. The CSA-dose response curves of NAP activity were linear. These findings suggest a close relationship between the two activities but do not prove that these activities are derived from a single biological substance, ie, the CSF. Sato et al[17] also demonstrated strong NAP-inducing activity in the cystic fluid of transplanted human squamous cell carcinoma, which is known to produce a large amount of CSF. According to Nicola et al,[18] HPCM contains two types of CSF; CSFα may act as GM-CSF and EO-CSF, and CSFβ as G-CSF.[18] Abboud et al[19] also separated two types of CSF from GCT-CM. Even if NAP-inducing activity is the same as CSA, it is necessary to clarify what kind of CSF has the former activity as well. The possibility remains that NAP-inducing activity unrelated to CSF is released from CML monocytes and is present in the CSA sources.

The biochemical and functional aspects of neutrophils, such as phagocytosis and NAP activity,[20] adhesiveness, and lactoferrin contents,[21] have been found to be closely related. Because these characteristics appear to develop together and to become most marked in the segmented forms,[1] NAP can be regarded as a marker enzyme for neutrophilic maturation. Lactoferrin, another marker of neutrophilic maturation, was reported to be induced in a dose-dependent manner by addition of exogenous CSA.[22] Furthermore, there is an increasing number of reports that CSF can stimulate the functions of mature neutrophils.[23,24] Weisbert et al[23] showed that purified human GM-CSF enhances oxidative metabolism of mature neutrophils and demonstrated that the GM-CSF is identical to the neutrophil migration inhibition factor (NIF).[24] In this context, it is interesting to clarify the relationship between NAP-inducing activity and CSFs.

In the liquid culture, monocyte depletion completely eliminated NAP induction in normal bone marrow neutrophils as well as in CML neutrophils. Thus both CML and normal neutrophils react similarly to the reduction of NAP-inducing factor derived from monocytes in the culture. In contrast to

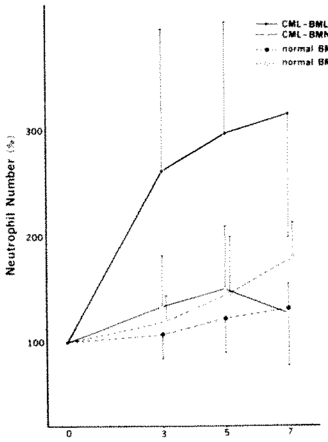

Fig 8. Changes in neutrophil number with time in liquid culture. Values are expressed as the percentage of increase from the initial neutrophil number of each sample. The bone marrow (BM) samples were obtained from three patients with chronic myelogenous leukemia (CML) and three normal volunteers. LD-cell, light-density cell; NP-cell, nonphagocytic cell.

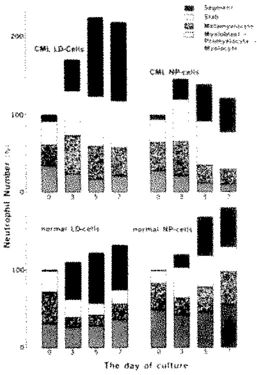

Fig 9. Changes in neutrophil numbers and cell compositions of bone marrow light-density (LD) and nonphagocytic (NP) cells with time in a representative case of chronic myelogenous leukemia (CML) and a normal volunteer. Values are expressed as the percentage of increase from the initial neutrophil number.

CML neutrophils, LD cells from normal bone marrow showed no significantly higher proliferative activity than did NP cells. It is not clear at present why NP cells can proliferate at a similar rate with less endogenous CSA. The higher percentage of immature neutrophils capable of division in the NP cell sample seems to play a role in this phenomenon.

Judging from the results of previous reports and the present study, the low NAP activity of CML neutrophils does not seem to be an intrinsic defect due to their leukemic nature. However, it is still obscure why the NAP activity is uniformly and markedly low in vivo at the time of diagnosis. In theory, some inhibitory activity against NAP-inducing activity may exist in vivo. Patients' sera, plasma, conditioned media of mature neutrophils, and neutrophil debris were tested for their inhibitory effect on NAP-induction in autologous CML LD cell culture, but they were not inhibitory (data not shown). NAP-inducing activity and probably CSA may be low in vivo. Because, in this study, CML monocytes showed distinct NAP-inducing activity as well as CSA in vitro, and that CSA of the conditioned media of the bone marrow[25] and the urine[26] from CML patients are not low compared with normal controls, it is unlikely that these activities are absent in vivo.

There are also some clinical observations concerning the mechanism of low NAP activity in vivo. Kamada and Uchino[27] observed during the preclinical phase of CML that NAP activity actually begins to decrease after peripheral leucocytosis is established. It is also well known that NAP activity increases after successful reduction of myeloid hyperplasia by chemotherapy, although the Ph^1 clone persists. At the time of diagnosis, CML bone marrow usually consists of an extraordinarily large number of neutrophils. Subsequently, the ratio of monocytes to neutrophils becomes low compared with normal bone marrow. NAP induction in vivo may be closely related to the degree of myeloid hyperplasia. The amount of the NAP-inducing activity released by monocytes available to individual neutrophils may decrease after the hyperplasia is established. The amount of CSA in individual neutrophils may also be low. The consistent elevation of NAP activity in vitro can be explained by the higher ratio of monocytes in the LD cell sample than in vivo and the closed circumstance of 1 mL liquid culture where the neutrophils (target cells) and the monocytes/macrophages (effector cells) are kept in close contact. The low NAP activity of CML neutrophils seems to result from extreme myeloid proliferation, which might be induced by a more fundamental pathogenesis, such as recently postulated mechanism of altered c-abl protooncogene transcription due to the Ph^1 translocation.[28]

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I wish to thank Drs Michito Ichimaru and Masao Tomonaga for helpful discussion through this study and for reviewing the manuscript, and Drs Kazutaka Kuriyama, Masuko Tagawa, Itsuro Jinnai, Tohru Jubashi, Yoshiharu Yoshida, Tatsuhiko Amenomori, and Hiroaki Nonaka for their cooperation.

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Biochemical Properties of the Eosinophil Cationic Protein and Demonstration of Its Biosynthesis In Vitro in Marrow Cells From Patients With an Eosinophilia

By Inge Olsson, Ann-Maj Persson, and Ingemar Winqvist

The eosinophil cationic protein (ECP), which has been shown to be secreted both in vitro and in vivo, is a cytotoxic unique constituent of eosinophil granules. To increase the understanding of the mechanisms behind the role of the eosinophil as a cytotoxic effector in disease, a detailed biochemical characterization of ECP was performed. A considerable molecular heterogeneity was revealed when purified ECP was eluted isocratically from a high-resolution cation exchange resin; the separation, reproducibly achieved, of five components was probably due to hydrophobic interaction with the resin. These polypeptides, which reacted quantitatively with anti-ECP antiserum, showed molecular weights (mol wt) of 19,500 and 16,700 and showed almost identical amino acid compositions. The amino-terminal sequence for one of the polypeptides was (in the standard one-letter code) (R-P-X-Q-F-T-R-A-Q-W-F-A-I-Q-H-I-S-L-N-P-R-R-C-T-I-A-M-R-A-I-N-N-Y-). The biosynthesis of ECP was demonstrated in marrow cells from patients with eosinophilia using labeling with (^{14}C)-leucine, followed by immunoprecipitation with anti-ECP, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and fluorography for visualization of labeled ECP. Biosynthesis was demonstrated of mol wt 22,000 ECP, which may represent precursor ECP, since with time some of it was processed into ECP with a mol wt of 18,000 to 19,000. Monensin, a proton ionophore, blocked the processing of mol wt 22,000 ECP. This study shows that ECP consists of a family of similar polypeptides. These may, however, have different biological activities.

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THE HUMAN EOSINOPHIL GRANULE is characterized by its rich content of highly cationic proteins. Among these are the major basic protein (MBP),[1,2] the eosinophil cationic protein (ECP),[3,4] and the eosinophil-derived neurotoxin (EDN).[5] MBP, ECP, and EDN represent distinctive cationic proteins of eosinophils as judged by their immunochemical and physicochemical properties.[6] After secretion, these strongly basic proteins are likely to be responsible, at least in part, for the cytotoxic effects of eosinophils on parasites[7,8] and cells,[9-11] although the specific effects have not yet been defined. Detailed characterization of the eosinophil cationic proteins should therefore lead to an understanding of the mechanisms behind their roles as cytotoxic effector molecules and perhaps to an understanding of the functions of the eosinophil.

ECP represents a family of immunologically identical extremely basic molecules[3,4] with an isoelectric point higher than pH 11. The major ECP constituent was reported to have a molecular mass of 21,000 daltons,[3,4] to contain 21 residues of arginine, and to be a zinc-containing protein. ECP does not show bactericidal or esterolytic activities[4] but damages schistosomula of *Schistosoma mansoni*[8] as well as cultured heart muscle cells.[11] Furthermore, it augments factor XII-dependent coagulation pathways,[12] which may be of importance to explain thromboembolic complications of hypereosinophilia.[13] No enzyme activity has been reported for ECP.

In this work, an extended purification and characterization of ECP was attempted by use of high-performance ion exchange chromatography and the resolution was achieved of homogeneous polypeptides suitable for amino acid sequence analyses. Mechanisms behind the biosynthesis, processing, storage, and secretion of granule proteins are largely unknown. Previous work with monoclonal antibodies has shown that one antibody recognized both storage and secreted forms of ECP, whereas another antibody only bound to ECP during secretion.[14] Thus solubilization of ECP in granules is associated with alterations in antigenicity. In the present work, we radiolabeled ECP biosynthetically in bone marrow cells from patients with eosinophilia. Cells were incubated with (^{14}C)-leucine, and ECP was isolated by immunoprecipitation, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography for visualization of newly synthesized ECP. The results indicated that ECP is subjected to alterations in size subsequent to biosynthesis.

MATERIALS AND METHODS

Materials. Sephadex G-75 superfine, CNBr-activated Sepharose 4B, protein A-Sepharose CL 4-B, and Mono S cation exchange columns were from Pharmacia, Fine Chemicals (Uppsala, Sweden). The Pharmacia fast-pressure liquid chromatography (FPLC) system was used for chromatography. Acrylamid/Bis 29:1 was obtained from Bio-Rad Laboratories (Richmond, Calif). L-(^{14}C)-Leucine (342 mCi/mmol) and En^{3}hance were from New England Nuclear (Boston). Phenylmethylsulfonylfluoride (PMSF), Triton X-100, and Monensin were from Sigma Chemical Co (St Louis). Diaflo membranes were from Amicon (Lexington, Mass).

Purification of ECP. ECP was isolated by methods previously described.[3,4] Briefly, leukocytes from a patient with chronic myeloid leukemia (CML) with 15×10^9 blood eosinophils per liter and leukocytes from a patient with bronchial carcinoma with 32×10^9 blood eosinophils per liter were homogenized in 0.34 mol/L sucrose, followed by differential centrifugation to obtain a crude granule fraction. Granules were extracted with 10 vol of 0.2 mol/L sodium acetate buffer, pH 4. After concentration by ultrafiltration on a Diaflow PM-10 membrane, chromatography was performed on a Sephadex G-75 column, and the material of the ECP-containing peak was further separated by ion exchange chromatography on E-aminocaproic acid Sepharose. These procedures resulted in the isolation of a family of essentially pure ECPs reacting with an antibody against the major ECP component, which had been purified by electrophoresis on agarose.[3,4] The antibody was produced by immunization of rabbits with the homogenous major ECP component as described previously.[3,4]

The isolated ECPs were analyzed by chromatography on a 1-mL

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Mono S cationic exchange column using the FPLC system. Elution was with a gradient of sodium acetate pH 5.0 at a flow rate of 1 mL/min.

Amino acid composition. Amino acid composition was determined with an automatic amino acid analyzer after hydrolysis in 6 mol/L HCl under argon at 110 °C for 24 hours.

Amino acid sequence analysis. Purified ECP was reduced and carboxymethylated with iodo(^{14}C)acetic acid in 6 mol/L guanidine-HCl. Automated amino acid sequence analysis was carried out by the method of Edman and Begg[15] on a Beckman 890C sequencer (Beckman Instruments, Palo Alto, Calif) by use of the standard Beckman 1M Quadrol program. All fragments were run in the presence of Polybrene.[16] Phenylthiohydantoin (PTH) derivatives of amino acids were identified by high-performance liquid chromatography (HPLC) on a 30-cm Waters u-Bondapak C18 column using stepwise elution with methanol-containing buffers for the ehylacetate phases and acetonitrile-containing buffers for the aqueous phases. PTH derivatives of amino acids were also identified by thin-layer chromatography.[17] PTH-carboxymethylcysteine residues were also identified by measurements of radioactivity.

Isolation of marrow cells for biosynthetic labeling of ECP. Bone marrow cells from five patients with eosinophilia were collected in heparinized McCoy's medium and erythrocytes removed by dextran sedimentation. The cells from the supernatant were washed and used for biosynthetic labeling. Four patients had the idiopathic hypereosinophilic syndrome with blood eosinophils in the range of 3 to 14 × 10^9/L and a duration of the illness from one to 13 years. One patient had eosinophilia secondary to a malignant lymphoma. Marrow cells were also isolated from one patient with chronic myeloid leukemia and 12 × 10^9 blood eosinophils per liter.

Labeling of cells. For biosynthetic labeling of ECP, marrow cells were starved in leucine-free minimum essential medium (Eagle) with 1% fetal bovine serum (FBS) for 60 minutes at 37 °C. The labeling medium was the same deficient medium with 10% FBS. The cells (3 × 10^6/mL) were incubated with 15 μCi/mL of (^{14}C)-leucine for one to 18 hours. In chase experiments, cells were first pulsed with 50 μCi/mL (^{14}C)-leucine for 60 minutes, washed and suspended in RPMI 1640 medium with 10% FBS with 10^6 cells per milliliter, followed by incubation for various chase time periods.

Extraction of cells. A radioimmunoprecipitation assay (RIPA) buffer consisting of 0.15 mol/L NaCl, 30 mmol/L HEPES, pH 7.3, 1% (vol/vol) Triton X-100, 1% (wt/vol) sodium deoxycholate, and 0.1% (wt/vol) sodium dodecyl sulfate (SDS) was used for extraction. Approximately 1 mL of RIPA buffer with 1 mmol/L PMSF was added per 10^7 cells and the extracts kept in ice for one hour, followed by centrifugation at 4 °C at 32,000 g for two hours. The clear supernatant was stored frozen until used for immunoprecipitation.

Immunoprecipitation of radioactive ECP. RIPA-buffer extracts, 100 to 300 μL, were mixed with 15 μL anti-ECP. After standing at 4 °C for 12 to 18 hours, 40 μL of a protein A-Sepharose in RIPA buffer (200 mg/mL) was added for collection of the immunoprecipitate by rotation at 4 °C for 60 minutes. The protein A-Sepharose was washed five times with RIPA buffer and the supernatant was carefully removed. The pellet was suspended in 50 μL H_2O with 15 μL electrophoresis sample buffer (0.4 mol/L Tris, pH 6.8, 50% glycerol, 10% SDS, and 5% β-mercaptoethanol), boiled for five minutes, and used for electrophoresis.

SDS-PAGE and fluorography. SDS-PAGE was performed on slab gels 18 cm long, 1.5 mm thick, and 16 cm wide, with ten wells, in an LKB 2001 Vertical Electrophoresis unit (LKB Products, Bromma, Sweden) according to Laemmli.[18] Samples were loaded on a linear gradient of 5% to 20% polyacrylamide gel with 3% stacking gel. The electrophoresis was run at 25 mA per gel without cooling. After electrophoresis, gels were fixed in 10% trichloroacetic acid, 10% acetic acid, and 30% methanol for at least one hour and treated with En^{3}hance for one hour and H_2O for one hour. Gels were dried on filter paper and exposed to x-ray film (Kodak X-Omat S, Eastman-Kodak Co, Rochester, NY) at −80 °C for three to six days.

Apparent molecular weights (mol wt) were determined by use of the following (^{14}C) methylated standards (New England Nuclear): cytochrome C, 12,300; carbonic anydrase, 30,000; ovalbumin, 46,000; bovine serum albumin, 69,000; and phosphorylase B, 97,400.

To quantitate radioactivity, individual bands, localized on the dried gel using fluorography, were excised and treated overnight at 37 °C in 1 mL of a solution containing 95 parts 30% H_2O_2 and five

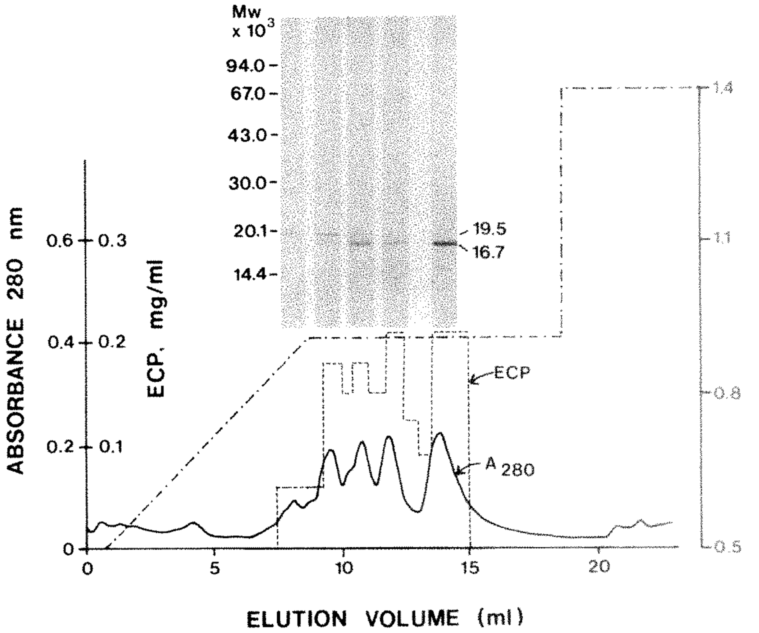

Fig 1. ECP, 1 mg, isolated from blood eosinophils of a patient with bronchial carcinoma using gel chromatography and E-aminocaproic acid Sepharose chromatography, was subjected to high-resolution chromatography on Mono S using the FPLC system. Elution was with sodium acetate pH 5.0 (—·—·) at a flow rate of 1 mL/min. The absorbance at 280 nm is monitored, as well as the ECP content of fractions determined by radial immunodiffusion (----). The SDS-PAGE pattern of ECP eluted in various fractions is also included, with mol wt markers.

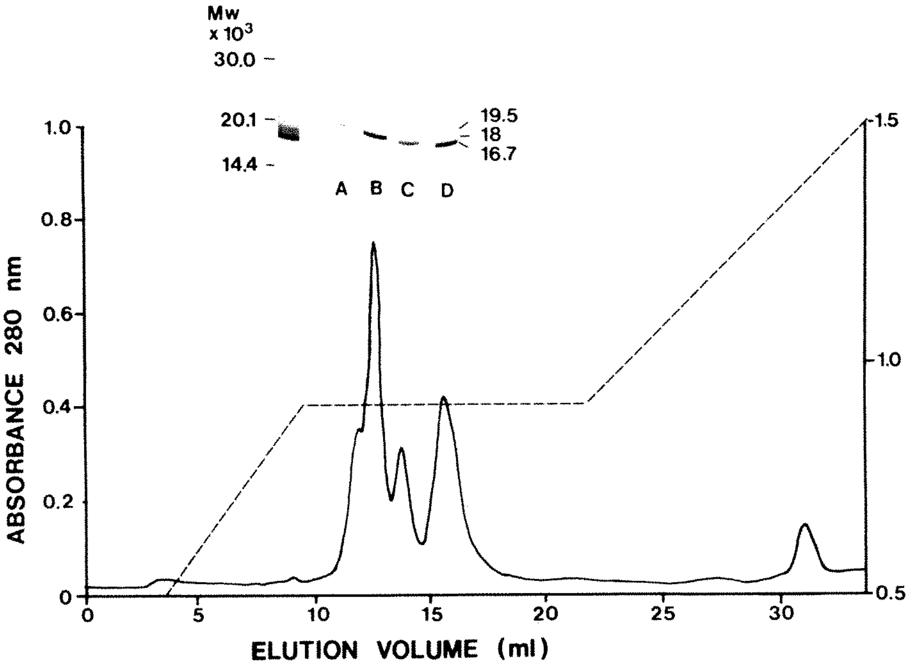

Fig 2. ECP, 2.5 mg, isolated from blood leukocytes of a patient with CML using gel chromatography and E-aminocaproic acid Sepharose chromatography, was subjected to chromatography on a Mono S column. Elution was with sodium acetate pH 5.0 (----) at a flow rate of 1 mL/min. The absorbance at 280 nm is monitored. The SDS-PAGE pattern of ECP eluted in different peaks (A through D) is included, as well as the SDS-PAGE pattern of the ECP lot used for the Mono S chromatography and mol wt markers.

parts 30% NH_3. Fifteen milliliters of scintillation liquid was added for counting.

RESULTS

High resolution ion exchange chromatography of ECP. ECP isolated by gel filtration, followed by E-aminocaproic acid Sepharose chromatography from blood eosinophils of a patient with bronchial carcinoma and high eosinophilia showed two distinct components when analyzed by SDS-PAGE. The mol wt of these components were 19,500 and 16,700. All ECP was eluted from a Mono S column with approximately 1.0 mol/L sodium acetate, pH 5.0, without achieving separation of the ECP components. Surprisingly, however, a considerable heterogeneity was revealed by isocratic elution with 0.9 mol/L sodium acetate, which resulted in the resolution of five separate components (Fig 1). As demonstrated in Fig 1, all components reacted quantitatively in a radial immunodiffusion assay using an antiserum raised against one homogeneous ECP species isolated by preparative electrophoresis.[3,4] Thus a marked microheterogeneity for

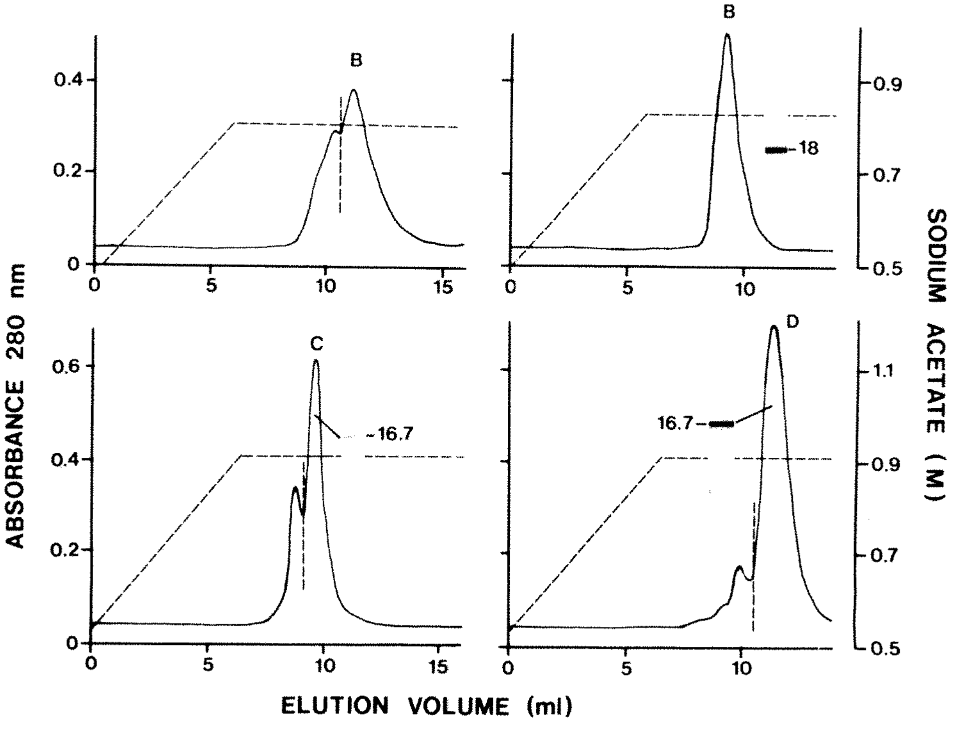

Fig 3. Rechromatography on Mono S of ECP components B, C, and D of the chromatogram shown in Fig 2. Elution was with sodium acetate pH 5.0 (----) at a flow rate of 1 mL/min. The absorbance at 280 nm is monitored. Component B was rechromatographed twice (upper part of figure). The SDS-PAGE pattern is included. As shown, components B and D were purified to homogeneity and component D was used for amino acid sequence analysis.

Table 1. Amino Acid Composition of ECP Components

	A	B	C	D
Aspartic acid	16.4	16.6	16.4	15.4
Glutamic acid	7.4	5.9	6.7	5.8
Glycine	6.2	4.4	5.1	5.0
Alanine	7.7	6.3	7.1	7.1
Valine	8.0	7.4	8.2	8.0
Leucine	5.2	6.0	6.4	6.5
Isoleucine	5.3	6.2	6.2	6.2
Serine	6.2	4.5	4.8	4.3
Threonine	6.0	5.8	5.8	5.6
Methionine	—	—	—	—
Proline	9.8	9.3	8.8	11.6
Phenylalanine	3.1	4.6	4.4	4.3
Tyrosine	0.6	1.7	0	2.0
Histidine	4.0	4.2	4.3	4.1
Lysine	1.1	1.1	1.0	0.9
Arginine	12.9	14.8	14.8	14.9

ECP components A through D were separated by high-resolution ion exchange chromatography (Figs 2 and 3). Data are given as residues/100. Cysteine and tryptophan were not quantitatively determined. Methionine was barely detectable, but the amino acid sequencing of the NH_2-terminus of component D revealed one methionine residue.

ECP molecules was found. ECP in the first two peaks eluted from the Mono S column had a mol wt of 19,500, while that of the three peaks eluted later had a mol wt of 16,700 (Fig 1). The elution pattern of ECP was reproducible, and an identical separation pattern as in Fig 1 was seen in another experiment with ECP purified from eosinophils of a patient with the idiopathic hypereosinophilic syndrome (data not shown). Eosinophils from a patient with CML were used for detailed biochemical characterization of ECP, which was obtained from the Mono S column in four peaks by isocratic elution with 0.9 mol/L sodium acetate, pH 5.0 (Fig 2). By rechromatography twice of peak B, a homogeneous protein species was obtained with a mol wt of 18,000 (Fig 3). Rechromatography once of peak D resulted in a homogeneous polypeptide with a mol wt of 16,700 (Fig 3). ECP in peak C was difficult to isolate in a homogenous state by rechromatography.

Amino acid analyses. Table 1 shows amino acid analyses of ECP components A through D isolated by high-resolution ion exchange chromatography (Figs 2 and 3). The amino acid compositions were almost identical for all ECP components.

Amino acid sequence of ECP. The amino acid sequence of the NH_2-terminus is shown in Fig 4. Thirty-three amino acid residues were sequenced. The third residue could not be identified.

Biosynthesis of ECP. Marrow cells from a patient with CML and a patient with an eosinophilia were incubated with (^{14}C)-leucine for different times, followed by extraction and immunoprecipitation with anti-ECP and SDS-PAGE. The fluorograms (Fig 5A and B) showed that ECP polypeptides were synthesized with a mol wt of 22,000. With time, CML marrow cells produced polypeptides with mol wt of 25,000 and 18,000. In addition, eosinophil marrow cells were pulsed with (^{14}C)-leucine for one hour and the label was chased for 1, 3, 7, and 18 hours and the fluorograms obtained after SDS-PAGE are shown (Fig 5C). The pulse-chase labeling experiments showed that some of the newly synthesized mol wt 22,000 ECP polypeptide was slowly converted into ECP with a mol wt of approximately 19,000. An experiment was also performed with these cells in an identical manner but with inclusion of 1 μmol/L Monensin during chase (Fig 5D). Monensin, a carboxylic proton ionophore that exchanges preferentially Na^+ ions for protons, prevented the conversion of mol wt 22,000 ECP into lower mol wt products.

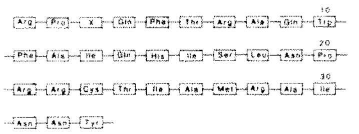

Fig 4. The NH_2-terminus amino acid sequence of ECP (component D of Fig 3).

The time course for incorporation of (^{14}C)-leucine into mol wt 22,000 ECP is shown in Fig 6. Incorporation increased for at least 15 hours.

Figure 7 shows results from ECP biosynthesis studies of marrow cells in five patients with eosinophilia. In all patients, the major ECP product had a mol wt of 22,000, while a minor product had a mol wt of approximately 18,000. Monensin prevented the formation of the mol wt 18,000 ECP. However, total biosynthesis of ECP was inhibited by Monensin to a variable degree. Therefore, the conclusion that Monensin diminished the production of immunoprecipitable ECP species with mol wt of less than 22,000 is not unambiguous from these data. However, data from the pulse-chase experiment of Fig 5D clearly show that Monensin prevents formation of ECP with a mol wt of 18,000 to 19,000.

DISCUSSION

One approach to the understanding of eosinophil function is to study the physicochemical properties and biological actions of eosinophil granule constituents. Eosinophil cationic polypeptides of granules may be responsible for cytotoxic effects of eosinophils on parasites and cells. In this work we show that an extended separation of purified ECP revealed a considerable molecular heterogeneity. Thus by isocratic elution from a high-resolution cation exchange resin, five ECP components were resolved. The separation was probably not a result of ion exchange, but more likely a result of partition chromatography due to hydrophobic interactions between ECP and the resin. Although the different components showed slight differences in molecular mass judging from SDS-PAGE, the amino acid compositions were almost identical.

The separation pattern of ECP components on the cation exchange resin was highly reproducible, and ECP purified from different patients with eosinophilia showed an identical elution profile. Future work will show if the ECP components obtained differ in biological activities.

The sequence determination of the amino-terminal part of one ECP component revealed a unique sequence. Thus when this sequence was aligned with known protein sequences in a computerized search, no specific homologies were found.

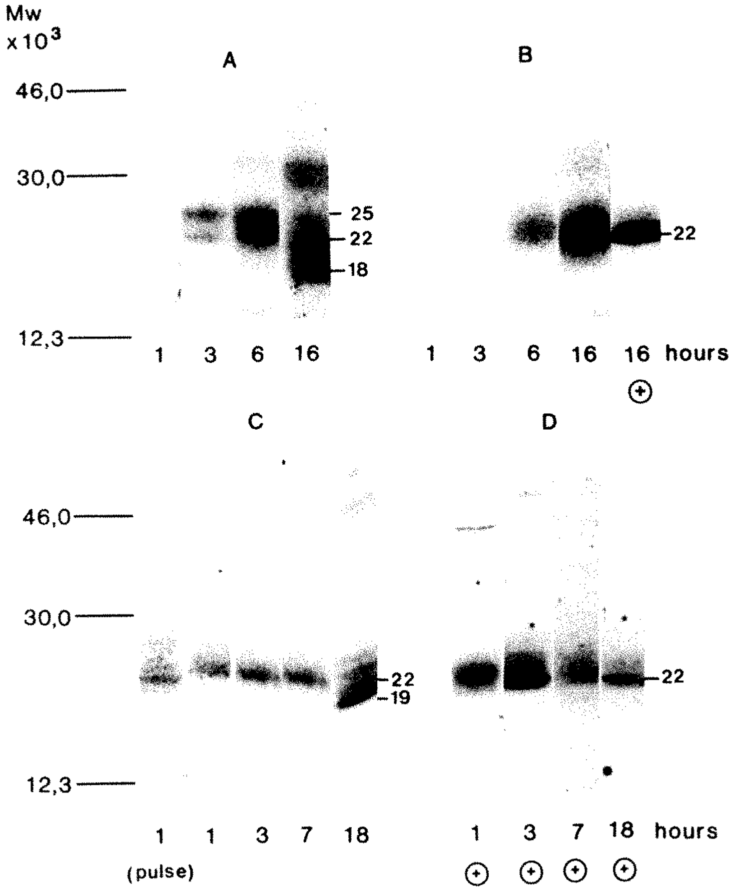

Fig 5. Biosynthetic labeling of ECP. Marrow cells from a patient with CML(A) or from patients with the idiopathic hypereosinophilic syndrome (B, C, and D) were used. In A and B, cells were incubated for various times with (^{14}C)-leucine, followed by extraction, and immunoprecipitation with anti-ECP and SDS-PAGE. In C and D, cells were pulse-labeled with (^{14}C)-leucine for one hour and the label was chased for up to 18 hours. Some incubations were performed in the presence of 1 µmol/L Monensin ⊕. Mol wt markers are shown to the left; mol wt of major ECP components are also shown.

Minor homologies with the receptor-binding site of diphtheria toxin (constituting eight of the sequenced 33 amino acids of ECP) are probably without significance.

In the present study, ECP was purified from granule extracts. Eosinophils have an ability to secrete ECP both in vitro[19] and in vivo.[20] It is not known, however, whether all forms of ECP, as detected in the present work, are secreted to the same extent. The secretory process has been investigated by the use of monoclonal antibodies against ECP.[14] One antibody to ECP bound to eosinophils from both healthy individuals and patients with eosinophilia. A second antibody also recognized ECP but was specific for the secreted form of the protein. The results suggest that there are structural alterations of ECP during secretion.

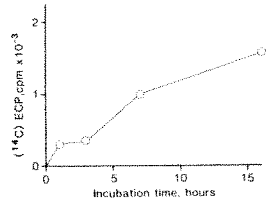

Fig 6. Time course for incorporation of (^{14}C)-leucine into ECP. Marrow cells were incubated for various times with (^{14}C)-leucine (corresponding to B in Fig 5), followed by extraction, and immunoprecipitation with anti-ECP and SDS-PAGE. Individual bands corresponding to the mol wt 22,000 polypeptide were localized on the dried gel using fluorography, excised, and counted by liquid scintillation. Each time point represents ECP labeling of approximately 10^7 eosinophils at various stages of maturation.

In another approach to study structural modification of ECP, biosynthetic labeling was used, followed by immunoprecipitation with anti-ECP. Marrow cell specimens from patients with eosinophilia were used for these experiments. All experiments demonstrated biosynthesis of ECP with a mol wt of 22,000. This form does not correspond to any of the ECP forms purified from eosinophil granules and may therefore represent a precursor polypeptide for ECP, which is subject to processing during intracellular transport and storage in eosinophil granules. It is consistent with this interpretation that some lower-mol-wt ECP appeared with time. This was most clearly shown in pulse-chase labeling experiments. The processing of mol wt 22,000 ECP into lower-mol-wt ECP was, however, not very rapid. In CML cells, but not with nonleukemic cells, production of immunoprecipitable ECP species with higher mol wt than 22,000 were seen. The significance of this finding is not clear.

The smallest polypeptide, mol wt 16,700 of purified ECP, which was the major form, was not biosynthetically labeled by incubation of cells in vitro for 16 hours. One explanation is that processing is slow and production of mol wt 16,700 ECP takes longer than 16 hours. Another explanation, although unlikely, is that proteolytic modifications took place during extraction and purification of ECP and that the mol wt 16,700 polypeptide is a degradation form of ECP.

As judged by pulse-chase labeling, Monensin, a proton

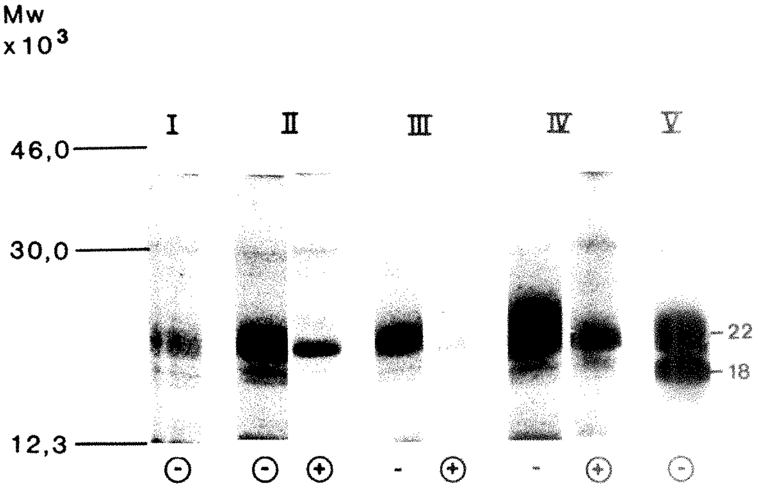

Fig 7. Biosynthetic labeling of ECP. Marrow cells from four patients with the idiopathic hypereosinophilic syndrome (I through IV) and one patient with eosinophilia secondary to malignant lymphoma (V) were used. Cells were incubated for 16 hours with (^{14}C)-leucine, followed by extraction, and immunoprecipitation with anti-ECP and SDS-PAGE. Cells were incubated without ⊖ or with ⊕ 1 μmol/L Monensin. Mol wt markers are shown to the left; mol wt are also indicated for labeled major ECP components.

ionophore, blocked the processing of newly synthesized ECP. This could be explained by the known inhibitory effects of Monensin on transport of secretory or lysosomal proteins and related to Monensin's ability to raise the normally acidic pH of the vacuolar system of the cell.[21]

Additional structural studies of ECP will require the use of DNA hybridization probes complementary to ECP-mRNA. Knowledge of the amino-terminal sequence of ECP can be used for synthesis of corresponding oligonucleotides to be used as probes for screening of a cDNA-library established from marrow cells of patients with an eosinophilia.

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An Abnormal Plasma Distribution of Protein S Occurs in Functional Protein S Deficiency

By Philip C. Comp, Deborah Doray, Dean Patton, and Charles T. Esmon

Protein S is a natural anticoagulant present in the plasma that serves as a cofactor for activated protein C. Patients deficient in protein S are subject to recurrent venous thrombotic disease. Protein S deficiency differs from other plasma protein deficiencies in that deficient patients often have normal or only mildly reduced levels of protein S in their plasma as detected by conventional immunologic methods but have markedly reduced functional protein S levels. This apparent discrepancy is due to the presence of two forms of protein S in plasma. The protein S is present free and in a complex with C4b-binding protein. The free form is functionally active, whereas the bound form is not. Examination by crossed immunoelectrophoresis of 31 functionally protein S–deficient individuals from seven families reveals that 29 of the 31 have all or most of their protein S complexed to C4b-binding protein with little or no free protein and have correspondingly low levels of protein S functional activity (type I deficiency). Two related protein S–deficient individuals show a different type of distribution with little or no protein S, either bound or free (type II deficiency). The detection and classification of protein S–deficient individuals requires the application of both a functional assay and an assessment of protein S distribution between bound and free forms.

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PROTEIN S IS A plasma protein that serves as a cofactor for the anticoagulant effects of activated protein C.[1] Patients deficient in protein C[2] and protein S[3-5] are prone to recurrent thromboembolic disease, presumably due to an inability to regulate the clotting cascade.

Measurement of circulating levels of these two natural anticoagulants is of interest in the evaluation of patients with thrombotic disease. Determination of protein C levels may be performed using Laurell rocket immunoelectrophoresis,[1] radioimmunoassay,[6] or functional assays.[7] Determination of protein S levels is complicated by the fact that protein S exists in plasma as the free protein and in reversible complex with C4b-binding protein, a regulatory protein of the complement system.[8] Laurell rocket immunoelectrophoresis has been used to determine plasma protein S levels.[3,4] However, with conventional immunoelectrophoresis, quantitative estimates of the distribution of protein S between free and bound forms have not proved reliable.[9] This is of particular importance in the determination of protein S levels since the protein S bound to C4b-binding protein does not serve as a cofactor for activated protein C.[3] An approach that has been used successfully to determine the relative distribution of complexed proteins in plasma is crossed immunoelectrophoresis.[10] We have determined that this method does in fact resolve the free from bound protein S in plasma and permits us to correlate functional protein S activity with immunologic levels of the free protein S.

The utility of a means of correlating functional and immunologic levels of protein S becomes clear when the functional assay we have employed is examined.[3] The assay is based on the potentiation of the anticoagulant effect of activated protein C by protein S, after clotting is initiated by the addition of factor Xa. The assay is sensitive to the level of protein S in the plasma. However, the assay involves the addition of undiluted patient plasma to the test system, and the base-line clotting time, as well as the response to activated protein C, is influenced by the anticoagulant status of the patient, liver failure, and nonspecific circulating anticoagulants, as well as sample collection and storage techniques. These limitations of the present assay technique make it essential to have an independent means of measuring protein S, both to confirm decreased levels of free protein S in patients with reduced functional protein S levels and to permit analysis of plasma samples that are not suitable for analysis by the functional assay.

MATERIALS AND METHODS

Reagents. Reagents were purchased from sources previously recorded[3] and were the highest grade commercially available.

Protein purification. Human plasma was the generous gift of the Oklahoma Blood Institute. Protein S and protein C were purified and protein C was activated as previously described.[3] Protein S functional activity was determined as previously described.[3]

Laurell rocket electrophoresis. Laurell rocket electrophoresis was conducted as previously described[3] using an LKB Multiphor electrophoresis chamber (LKB Instruments, Bromma, Sweden) with the cooling plate set at either 10 °C or 25 °C as indicated in the text.

Crossed immunoelectrophoresis. Crossed immunoelectrophoresis was conducted using the method of Laurell.[10] Electrophoresis in the first dimension was conducted at 10 °C at 6 mA/cm for one hour. The agarose gel contained 2 mmol/L ethylenediaminetetraacetic acid (EDTA) in Paragon B-2 barbital buffer (Beckman Instrument Company, Brea, Calif). At the end of one hour the agar plate was removed from the chamber and the excess gel removed. The second-dimension gel containing 7 mmol/L EDTA, 2% polyethylene glycol (PEG) 8000 (Lot B14A, Eastman Kodak, Rochester, NY) in Paragon B-2 buffer with 0.1 mg/mL goat anti–protein S IgG was poured onto the plate. The second-dimension electrophoresis was conducted at 25 °C with 2 mA/cm for 18–20 hours.

Electrophoretic determination of the distribution of protein S. Protein S complexed to C4b-binding protein is precipitated at 4 °C when PEG 8000 is added to plasma to a final concentration of

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3.75%. Free protein S is not precipitated under these conditions and may be quantitated using Laurell rocket electrophoresis. The C4b-binding protein–protein S complex was precipitated as follows: PEG 8000 was dissolved in distilled water to give a final concentration of approximately 250 mg/mL. (The exact concentration of PEG 8000 was determined by drying a 1-mL aliquot of the stock solution before proceeding). A 100-μL sample of plasma was pipetted into the bottom of a 400-μL disposable micro–test tube (Bio-Rad Laboratories, Richmond, Calif), and the sample was warmed in a 37 °C water bath for 5 minutes. PEG 8000 stock solution was added to give a final concentration of 3.75% in plasma; the tube was capped and mixed in an inverted position on a Vortex-Genie mixer (Scientific Instruments, Inc, Bohemia, NY), set at medium speed, for 5 seconds. The tube was then turned upright and the mixing was repeated. Both the inverted and upright mixing steps were repeated. The sample was immersed in melting ice water for 30 minutes. The precipitate was removed by centrifugation in a Beckman microfuge B (Beckman Instruments) for 2 minutes at room temperature. The supernatant was analyzed by Laurell rocket electrophoresis conducted at 10 °C. All plates included a standard curve that consisted of the supernatant from pooled normal plasma undiluted and diluted 1:1, 1:2, and 1:4 with electrophoresis buffer. Patient values obtained were expressed as a percentage of the normal pool. The between-day variation and within-day variation were both 7%. To determine recovery of free protein S in the supernatant, the starting plasma was supplemented with ^{125}I-labeled protein S. The labeled protein S was fully functional following labeling as determined in the functional assay. To minimize binding of ^{125}I–protein S to C4b-binding protein, the PEG 8000 precipitation was carried out as outlined immediately after addition of the radio-labeled protein S. Greater than 95% of the exogenous ^{125}I–protein S remained in the supernatant following this precipitation. The concentration of 3.75% PEG 8000 was selected on the center of a range of PEG 8000 concentrations in which the level of free protein S remaining in the supernatant remains constant (Table 1).

Protein S–deficient patients. The families of four previously identified unrelated protein S–deficient individuals were studied. All protein S–deficient individuals had functional protein S levels less than 43% of normal and had free protein S levels of less than 40% as estimated by quantitative crossed immunoelectrophoresis.[11] None of the patients studied were receiving heparin, and none had received oral anticoagulants for at least one month. The plasma of more than 80% of the patients was examined on more than one occasion.

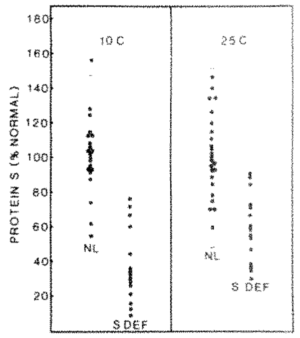

Fig 1. Laurell rocket electrophoretic analysis of protein S antigen levels determined in normal and protein S–deficient individuals. Rocket immunoelectrophoresis was conducted at 10 °C (left panel) and 25 °C (right panel) under the conditions indicated in the text.

Table 1. Effect of PEG 8000 Concentration on Concentration of Protein S Remaining in Plasma Supernatant

PEG 8000 (%)	Rocket Height (mm)
1.5	17.0
2.0	17.1
2.5	16.8
3.0	8.3
3.5	8.0
4.0	7.9
4.5	6.9
5.0	5.0
6.0	4.1

PEG 8000 precipitation was conducted as described in the text, and rocket immunoelectrophoresis was carried out at 25 °C. The free protein S concentration remaining in the supernatant following PEG 8000 precipitation remained constant from approximately 3.5% to 4.0% PEG 8000. No complexed protein S was seen by crossed immunoelectrophoresis at these concentrations; the size of the free protein S peak did not appreciably change over this range as judged by crossed immunoelectrophoresis.

RESULTS

When patient plasma is analyzed by Laurell rocket electrophoresis, free and bound forms of protein S cannot be distinguished. If electrophoresis conditions that slow the dissociation of the C4b-binding protein–protein S complex (10 °C, low voltage) are employed, 27% of the protein S–deficient patients fall within the normal range (Fig 1). If conditions are altered to promote dissociation of the complex, either before electrophoresis (by addition of urea to the sample) or during electrophoresis (by raising the temperature to 25 °C or by prolonging electrophoresis time), 65% of the patients appear to have normal protein S levels (Fig 1). This dependence of the observed protein S level on the electrophoresis conditions suggested that this method of quantitation of protein S might prove unsatisfactory.

Dahlbach and Stenflo had demonstrated that crossed immunoelectrophoresis can successfully resolve bound and free forms of protein S,[8] and this method was applied to plasma samples (Fig 2). In normal plasma from normal individuals, two distinct peaks reacting with antibodies against protein S are observed. The protein S associated with C4b-binding protein remains near the origin, whereas the free protein S migrates farther toward the positive electrode in the first dimension.

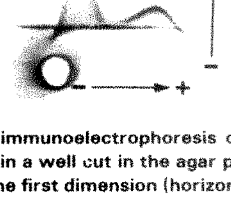

Fig 2. Crossed immunoelectrophoresis of plasma. A plasma sample was applied in a well cut in the agar plate and electrophoresis conducted in the first dimension (horizontal); agar containing antibodies directed against protein S was then poured above the first gel, and electrophoresis was conducted in the second dimension. Lines of precipitation show free plasma protein S (right peak) and protein S associated with C4b-binding protein (left peak).

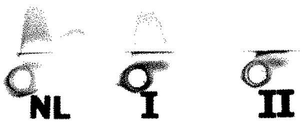

Fig 3. Characterization of protein S deficiency by crossed immunoelectrophoresis. The crossed immunoelectrophoretic pattern of normal plasma is on the left (NL). The crossed electrophoretic analysis of plasma from a 42-year-old female with less than 10% functional protein S activity is indicated as type I (center). This individual has protein S associated with C4b-binding protein but no evidence of free protein S. The individual designated type II (right) is the son of two individuals who have the type I pattern. This man has no free protein S, and little or no protein S is seen in the region of the C4b-binding protein S complex. His plasma has no detectable protein S functional activity. Of the functionally deficient patients examined, 94% had the type I pattern and 6% the type II pattern.

Using crossed immunoelectrophoresis, we examined the plasma of 31 individuals who were functionally deficient in protein S (Fig 3). Twenty-nine of the protein S–deficient patients have little or no free protein S but do have significant quantities of protein S associated with C4b-binding protein as is exemplified by the electrophoretic pattern of the patient designated type I. Two of the patients have no free protein S and very little protein S associated with C4b-binding protein and are designated type II. These data suggest that crossed immunoelectrophoresis might be of value in the determination of protein S since, in contrast to conventional rocket electrophoresis, crossed immunoelectrophoresis provides information on the relative distribution of protein S between free and bound forms.

However, crossed immunoelectrophoresis involves two separate electrophoretic steps and requires considerable technical expertise. Although the method can be employed for semiquantitative purposes,[11] it is relatively costly in terms of materials and performance time. We therefore sought a means of estimating only free protein S levels in plasma without using crossed immunoelectrophoresis.

Dahlback[12] found that the addition of PEG 8000 to plasma will precipitate the C4b-binding protein–protein S complex but not the free protein S. We confirmed the finding that addition of 3.75% PEG 8000 will precipitate the complex from plasma and leave the free protein S in the supernatant (Fig 4). Plasma samples may be treated in this manner and the free protein S in the plasma quantitated by rocket immunoelectrophoresis. The levels of free protein S remaining in the plasma supernatant following PEG precipitation are markedly different in normal and protein S–deficient individuals (Fig 5). While the total protein S levels (as measured by immunoelectrophoresis at 25 °C) may not be markedly decreased in type I protein S deficiency, the free protein S levels are decreased. The levels of free protein S determined using the PEG 8000 precipitation method and the functional protein S levels were compared in 28 normal individuals and in 15 protein S–deficient patients (Fig 6). There was a significant correlation between the free protein S levels and the functional protein S activity ($n = 0.81$, $P <$ .01). Normal free protein S levels estimated by the PEG precipitation method ranged from 57% to 120%, whereas the patient population ranged from < 5% to 43%.

To determine prospectively if this method of estimating free protein S levels might be of value in detecting protein S–deficient individuals, we investigated a large and previously undescribed family with a number of members who had recurrent venous thrombosis. The family tree is shown in Fig 7. Of the 81 members examined, 21 had low free protein S levels measured by the PEG precipitation method (Fig 6) with values ranging from < 5% to 41%. All the 21 family members predicted to be protein S–deficient did in fact have reduced functional protein S levels ranging from < 5% to

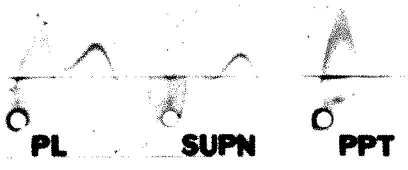

Fig 4. Crossed immunoelectrophoretic analysis of plasma, PEG 8000 supernatant, and precipitate. Starting plasma is shown at the left. The supernatant following PEG 8000 precipitation of the C4b-binding protein–protein S complex in the plasma (middle) and the resuspended PEG 8000 precipitate (right) are shown. A small peak of free protein S is seen in the right-hand panel and may reflect dissociation of the complex during resuspension and electrophoresis or trapping of free protein S in the precipitate. The decreased height of the free protein S in the supernatant reflects the dilutional effects of the added PEG 8000 solution added.

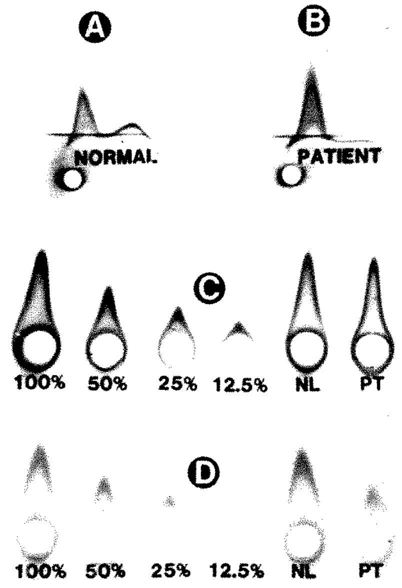

Fig 5. Rocket immunoelectrophoretic analysis reflecting total and free protein S antigen levels. In A and B are shown protein S distributions of a normal individual and a type I protein S–deficient individual. The immunoelectrophoresis analysis shown in C was performed against anti–protein S antibodies at 25 °C and reflects total protein S antigen levels (free and bound protein S). The analysis in D is of the PEG 8000 supernatant and reflects free protein S antigen. In both panels the samples were, from left to right, 100%, 50%, 25%, and 12.5% normal plasma, respectively; normal individual; and protein S–deficient individual. The deficient individual had total protein S levels of 80% but free protein S levels of approximately 21%.

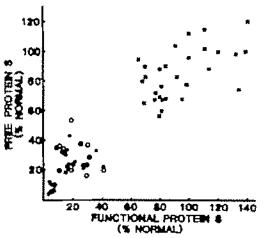

Fig 6. Levels of free plasma protein S antigen and functional protein S levels in normal and deficient individuals. Free protein S antigen levels and functional protein S levels were measured in normals (■), in 15 previously discovered protein S–deficient patients (●), and the newly discovered protein S–deficient family shown in Fig 7 (O).

41%. All had type I crossed immunoelectrophoretic patterns showing a reduction or absence of the free protein S peak. The incidence of venous thrombosis in these individuals was 62%. The other 60 family members had normal free protein S levels based on both the PEG precipitation method and crossed immunoelectrophoresis patterns. Of these 60 individuals, 5 had functional protein S levels below normal. Of these 5 patients, one was pregnant and another was on oral anticoagulants, both conditions that lower the observed functional level. One of these 60 family members with normal protein S status had a history of thrombophlebitis.

DISCUSSION

Protein S deficiency differs significantly from deficiencies of the other vitamin K–dependent plasma proteins and from factor VIII deficiency. The majority of protein S–deficient individuals appear to have normal or only moderately reduced total levels of protein S antigen in their plasma. However, the protein S that is present does not function as an anticoagulant. The explanation for this centers on the fact that the protein S that is present is bound to C4b-binding protein. Little or no free protein S is present in plasma. Free protein S correlates better than total protein S with functional protein S activity. This suggests that the functional protein S deficiency involves the quantitative distribution of protein S between free and bound form and not an abnormality of the protein S per se.

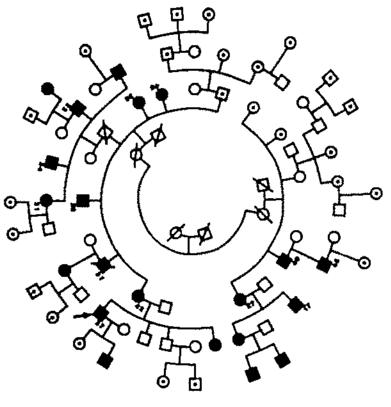

Fig 7. Protein S–deficient family. Family members were surveyed using the functional protein S assay and crossed immunoelectrophoresis. □, ○, individuals who were surveyed and found normal; ■, ●, protein S–deficient family members. Age of onset of thrombotic symptoms is indicated in subscript. The proband is indicated by the arrow.

Two brothers have been detected who have very little detectable protein S, either free or bound (type II). The parents of these men both have little free protein S but only mildly reduced levels of bound protein S. Why the sons have so markedly reduced levels of total protein S antigen remains to be determined, as does the question of why the distribution of protein S between free and bound forms is abnormal in the parents. Initial determinations suggest that C4b-binding protein levels in type I patients are elevated. This could provide an explanation for the shift in protein S from free to bound forms. The correlation of free protein S with C4b-binding protein levels will require better methods for detecting C4b-binding protein in plasma.

The mechanism of protein S deficiency remains to be elucidated. Until that occurs, we propose to designate the protein S–deficient patients with normal or mildly reduced total protein S antigen and markedly reduced free protein S as type I and those with markedly reduced total protein S antigen as type II. The inheritance of the type I defect is clearly autosomal dominant. The only type II individuals we have identified are sons of two type I–deficient individuals. It is likely that the sons inherited an abnormal gene from each of the parents.

Detection of protein S deficiencies is complicated. Conventional Laurell rocket electrophoresis should clearly identify the type II deficiency. However, this appears to be present in a minority of affected individuals. The combined use of a functional protein S assay and crossed immunoelectrophoresis or conventional rocket electrophoresis following PEG 8000 precipitation of complexed protein S appears to be the best means presently available of detecting these patients. The use of conventional rocket immunoelectrophoresis gives no indication of the distribution of protein S between free and bound forms and may tend to underestimate markedly the frequency of protein S deficiency.

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Elimination of Daudi Lymphoblasts From Human Bone Marrow Using Avidin-Biotin Immunoadsorption

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Biotinylated antibodies and an avidin-Sepharose 6MB column were utilized in a novel approach to deplete selected cell populations from human bone marrow. Fluorescein-labeled Daudi lymphoblasts were mixed with bone marrow mononuclear cells in a model system, and removal of Daudi cells was quantitatively assessed with an inverted fluorescent microscope. Treatment using the biotinylated monoclonal antibody 2H7 reactive with Bp32 antigen (expressed on Daudi cells) followed by passage over avidin-Sepharose produced greater than two logs of Daudi cell removal from bone marrow. An alternative method was tested by treating cells successively with nonbiotinylated monoclonal antibody and biotinylated goat antimouse immunoglobulin followed by passage over avidin-Sepharose. Up to three logs of Daudi cells could be eliminated from bone marrow with quantitative recovery of hematopoietic progenitors. The use of biotinylated goat antimouse immunoglobulin eliminates the need to prepare a biotin conjugate of each individual monoclonal antibody. The clinical application of cellular immunoadsorption using the avidin-biotin system may prove useful in bone marrow transplantation.

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BONE MARROW transplantation (BMT) is being used for treatment of increasing numbers of patients with hematologic malignancies, aplastic anemia, solid tumors, certain congenital hematologic disorders, and immunodeficiency states.[1,2] Graft-*v*-host disease (GVHD) in patients given allogeneic transplants and relapse possibly caused by the presence of residual tumor cells in autologous marrow transplants are among the major obstacles that limit the success of these procedures.[3–5] Investigators have demonstrated that GVHD can be prevented in animals by removal of T cells from the donor marrow before inoculation into the recipient.[6–8] Similarly, successful autologous marrow transplants have been performed in animals in which tumor cells were first removed in vitro from the donor marrow before injection.[9,10]

The successes of animal studies have led to multiple in vitro methods designed to eliminate T cells or tumor cells from human bone marrow.[11,12] These have included the use of antibodies with complement, antibody-toxin conjugates, various physical separation or fractionation procedures, chemotherapeutic agents, and magnetic immunoselection. We have previously described a cellular immunoadsorption technique that can eliminate T cells or tumor cells from bone marrow.[13,14] By using a column made of monoclonal antibodies directly linked to Sepharose 6MB gel, we have avoided the toxicity to hematopoietic precursors and decreased recovery of bone marrow sometimes observed with other in vitro techniques.

The large quantities of monoclonal antibodies required for direct attachment to Sepharose gel (5 mg/mL) have made scale-up of this procedure for clinical application to human BMT difficult. We have therefore developed an indirect cellular immunoadsorption column procedure that exploits the high affinity between the protein avidin and the vitamin biotin ($K_m = 10^{-15}$ mol/L).[15] We have accomplished the selective removal of Daudi tumor cells from human bone marrow mononuclear cells by using biotinylated monoclonal antibodies or nonbiotinylated murine monoclonal antibodies (reactive with Daudi cell surface antigens) and biotinylated goat antimouse antisera followed by passage over a column constructed with avidin linked to Sepharose 6MB gel. This system provides excellent recovery of bone marrow and preservation of committed hematopoietic stem cells.

MATERIALS AND METHODS

Antibodies

A purified murine monoclonal IgG2a antibody (2H7) to the human B cell differentiation antigen, Bp32(CD20), was kindly provided by Dr Jeff Ledbetter (Genetic Systems Corp, Seattle). A purified murine monoclonal IgG2a antibody (DM1) specifically recognizing the u heavy chain of human immunoglobulin was a gift of Dr Ronald Levy (Stanford University Medical Center, Stanford, Calif). The monoclonal IgG2a antibody 9E8, kindly provided by Dr Robert Nowinski, recognizes the p(15)E antigen of murine leukemia virus and is not reactive with human tissues, including bone marrow or Daudi tumor cells. Affinity purified goat antimouse immunoglobulin (GAMIg), biotinylated goat antimouse immunoglobulin (B-GAMIg), and biotinylated $F(ab')_2$ goat antimouse immunoglobulin (B-$F(ab')_2$-GAMIg) were obtained from Tago, Inc (Burlingame, Calif).

Biotinylation of Antibody 2H7

Antibody was dialyzed in 0.1 mol/L $NaHCO_3$ and adjusted to a concentration of 1 mg/mL. *N*-Hydroxysuccinimidobiotin (Sigma Chemical Co, St Louis) was dissolved in dimethylsulfoxide (DMSO) at a concentration of 1 mg/mL For most experiments, 60 μL of this solution were added to each milliliter of the antibody solution and incubated for four hours at room temperature. The mixture was then dialyzed in phosphate-buffered saline (PBS) and stored at 4°C.

Tumor Cell Line

Daudi is a human lymphoblastoid B cell line originally derived from a patient with Burkitt's lymphoma.[16] It was maintained in our laboratory by serial passage in tissue culture medium containing RPMI 1640 with 10% fetal calf serum, sodium pyruvate, L-

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glutamine, streptomycin, and penicillin. Indirect immunofluorescence staining and analysis using a fluorescence-activated cell sorter (FACS IV; Becton Dickinson, Mountain View, Calif) confirmed that 100% of the Daudi cells bear surface IgM. These studies also demonstrated that all cells expressed the pan-B cell marker Bp32(CD20). Jurkat is a T cell lymphoblastic leukemia line that does not express surface IgM or Bp32 antigens.[17]

Bone Marrow

Bone marrow samples (5 to 10 mL) were obtained from healthy donors with approval of the Human Subjects Committee at the Fred Hutchinson Cancer Research Center. Bone marrow mononuclear cells were obtained by Ficoll-Hypaque (sp gr = 1.077; Pharmacia, Piscataway, NJ) density gradient centrifugation

Avidin Conjugation to Solid-Phase Substrate

Cyanogen bromide–activated Sepharose 6MB (Pharmacia, Piscataway, NJ) was swollen for 35 to 45 minutes in 1 mmol/L. HCl. The gel was washed with additional 1 mmol/L HCl, followed by 0.1 mol/L $NaHCO_3$ in 0.5 mol/L NaCl at pH 8.3 (coupling buffer). Avidin (Sigma Chemical Co, St Louis) was dissolved and dialyzed in coupling buffer at a concentration of approximately 1 mg/mL and then continuously mixed with the gel for one hour at room temperature. The eluate fraction was collected, and the efficiency of conjugation, which always exceeded 95%, was determined by measurement of protein concentration. The concentration of avidin linked to the gel varied between 50 μg/mL and 2 mg/mL of gel. After washing the gel in an alternating fashion with 0.1 mol/L sodium acetate in 0.5 mol/L NaCl at pH 4.5 (washing buffer) and coupling buffer, 0.2 mol/L glycine in coupling buffer was added to block unconjugated sites. After washing, the gel was stored in PBS with 0.1% sodium azide at 4°C.

Preparation and Use of Column

Gel was packed in Pharmacia K9/15 columns (Pharmacia, Piscataway, NJ). In all experiments, 15×10^6 treated cells were suspended in 0.5 mL PBS containing 2% bovine serum albumin (PBS/BSA), passed through a 3-mL column bed of gel, and washed through with PBS/BSA at a continuous flow rate of 0.5 mL/min until a total of 10 mL of eluate was collected. Cell viability was judged to be greater than 98% by trypan blue dye exclusion. To regenerate the column, the gel was extensively washed with PBS (without bovine serum albumin) and mechanically agitated using a Pasteur pipette to dislodge adherent cells. The gel was then sequentially washed three times with washing buffer and coupling buffer and stored in PBS with 0.1% sodium azide at 4°C.

Treatment of Cells With Biotinylated Antibodies

Initial concentrations of antibodies used in these experiments were based on immunofluorescence staining and FACS analysis, which demonstrated that a minimum of 50 to 100 μg/mL of unconjugated or biotinylated antibody 2H7 or 5 to 10 μg/mL of antibody DM1 were required to saturate the binding sites for Bp32 or u on Daudi cells, respectively (data not shown).

Biotinylated antibody 2H7 Fifteen million cells/mL of bone marrow contaminated with 5% to 10% Daudi cells were incubated for 30 minutes with biotinylated antibody 2H7 in PBS/BSA at 4°C. Cells were then washed twice with PBS/BSA before column treatment.

Biotinylated goat antimouse antisera. Fifteen million cells/mL of a similar bone marrow and Daudi cell mixture were incubated for 30 minutes with either antibody 2H7 or antibody DM1 in PBS/BSA at 4°C. Cells were washed twice after incubation with the monoclonal antibody before and twice again after incubation with B-GAMIg or B-F(ab′)$_2$-GAMIg for 30 minutes at 4°C.

Labeling of Tumor Cells

Intracellular labeling of Daudi cells with fluorescein isothiocyanate (FITC) was performed using a modification of the procedure described by Butcher and Weissman.[18] Briefly, Daudi cells were incubated with 60 μg/mL FITC (Sigma Chemical Co, St Louis) in PBS for 20 minutes at 37°C. Cold PBS was added to stop labeling, and the cells were centrifuged through an underlayer of fetal calf serum to remove excess FITC. Cells were then washed twice with PBS. This method produces bright intracellular fluorescence in 100% of cells. Furthermore, cell viability assessed by trypan blue exclusion was identical in FITC-labeled Daudi cells compared to unlabeled Daudi cells. Immunofluorescence staining with rhodamine isothiocyanate–conjugated antibody 2H7 demonstrated that expression of Bp32 was unaffected by FITC labeling of Daudi cells.

Detection of Residual Tumor Cells

A known total number of bone marrow mononuclear cells and labeled Daudi cells was placed into 96-well flat-bottom microdilution plates (Costar, Cambridge, Mass) and examined with a Leitz inverted fluorescence microscope. A series of preliminary experiments demonstrated that a single FITC-labeled Daudi cell could be detected in a well containing a total of one million cells, representing a tumor cell contamination of 0.0001%. For most of the experiments described here, a pretreatment sample was collected and 1,000 cells placed in microdilution wells, while after column treatment, 100,000 cells were placed in these wells. The number of FITC-stained cells was counted in three separate wells and averaged to determine the number of tumor cells per 1,000 total cells before column treatment and the number of tumor cells per 100,000 cells in the posttreatment group, respectively. Total cell counts were performed with a hemacytometer. The log removal of Daudi cells was calculated from absolute cell counts and the relative percentage of FITC-labeled cells before and after column treatment as follows:

$$\log \frac{\begin{array}{c}\text{\% labeled cells in}\\ \text{pretreated sample}\end{array} \times \begin{array}{c}\text{total number of cells}\\ \text{in pretreated sample}\end{array}}{\begin{array}{c}\text{\% labeled cells in}\\ \text{treated sample}\end{array} \times \begin{array}{c}\text{total number of cells}\\ \text{in treated sample}\end{array}}$$

Percentage of recovery was determined as follows:

$$100 \times \frac{\begin{array}{c}\text{\% nonlabeled cells}\\ \text{in treated sample}\end{array} \times \begin{array}{c}\text{total number of cells}\\ \text{in treated sample}\end{array}}{\begin{array}{c}\text{\% nonlabeled cells in}\\ \text{pretreated sample}\end{array} \times \begin{array}{c}\text{total number of cells}\\ \text{in pretreated sample}\end{array}}$$

As an example, the calculations are illustrated from one of the experiments treating a mixture of bone marrow and FITC-labeled Daudi cells successively with 10 μg/mL antibody DM1 and 1:100 dilution of B-GAMIg and then passed over a 3-mL column bed of Sepharose containing 1 mg/mL of avidin. The pretreatment sample had a mean of 58 FITC-labeled cells (52, 59, and 62 labeled cells counted in three separate wells) per 1,000 cells (5.8%), while the posttreatment sample contained a mean of 21 FITC-labeled cells (19, 22, and 22 labeled cells counted in three separate wells) per 1.0×10^5 cells (0.021%). There were a total of 15.0×10^6 cells in the pretreatment sample and 11.4×10^6 cells in the posttreatment sample.

$$\text{Log removal} = \log \frac{(5.8\%) \times (15.0 \times 10^6)}{(0.021\%) \times (11.4 \times 10^6)} = 2.56$$

$$\text{Percentage of recovery} = 100 \times \frac{(99.98\%) \times (11.4 \times 10^6)}{(94.2\%) \times (15.0 \times 10^6)} = 80.7\%$$

Column Sterilization Procedure

The K9/15 column, including the rubber stopper and tubing, was sterilized with ethylene oxide. The column was then packed with gel and allowed to incubate for 30 minutes with 0.05% formaldehyde (vol/vol) in PBS Formaldehyde was removed by extensive washing with PBS. No loss of immunoadsorption efficiency occurred with use of this method of sterilization.

Assay System for Hematopoietic Progenitors

The assay system for culturing committed granulocyte-monocyte progenitors (CFU-C) was a modification of the method of Bradley and Metcalf.[19,20] Briefly, 1.0 × 10^5 cells were cultured in triplicate in 1 mL aliquot mixtures containing conditioned medium from phytohemagglutinin (Wellcome Labs, Oxford, England)-stimulated peripheral blood lymphocytes, agar (Bactoagon, Difco, Detroit), and MEM alpha medium (Gibco, Grand Island, NY) containing fetal calf serum. Colonies were counted after 14 days incubation at 37°C in 5% CO_2 using an inverted microscope.

RESULTS

Depletion of Daudi Cells With Biotinylated Antibody 2H7

Figure 1 summarizes the results from a series of experiments in which a mixture of bone marrow and Daudi cells was treated with 100 μg/mL biotinylated antibody 2H7 and then passed over a column containing Sepharose 6MB to which various amounts of avidin were linked. Increasing concentration of avidin on the gel was correlated with improved removal of Daudi cells. Avidin concentrations of 100 μg/mL or less resulted in less than one log depletion, while concentrations of 1,000 μg/mL were able to produce over two log removal of Daudi cells from bone marrow. However, increasing the concentration of avidin on the gel also caused decreased recovery of bone marrow cells.

Attempts to improve recovery of bone marrow were made by decreasing the biotin-antibody conjugation ratio (Table 1). Marrow recovery was increased, but tumor cell removal was decreased. Using another source of avidin (Vector Laboratories, Burlingame, Calif), altering elution fluid conditions such as pH or bovine serum albumin concentration, incubating cells with free biotin or unconjugated antibody (to block Fc receptors) did not improve bone marrow recovery. Passage of treated cells over a column containing Sepharose linked to Strepavidin (Sigma Chemical Co, St Louis), an avidin derivative reported to have less nonspecific binding properties than avidin, did improve recovery but resulted in less efficient removal of Daudi cells (data not shown).

Table 1. Effect of Amount of the Biotin-Antibody Conjugation Ratio on Removal of Daudi Cells From Bone Marrow

Concentration of Biotin Ester (μg/mL)	log Removal	% Recovery
60	2 10 ± 40	57 8 ± 4.6
30	1 49	72.9
20	1.55	82.5
10	0.90	86.0
5	0.64	96.4
1	0.26	89.3

Biotin-antibody conjugates were prepared by incubating antibody 2H7 (1 mg/mL) with the indicated concentrations of *N*-hydroxysuccinimidobiotin for four hours. Fifteen million bone marrow cells containing 5% to 10% Daudi cells were incubated for 30 minutes with 100 μg/mL biotinylated antibody 2H7 and passed over a 3-mL column bed of Sepharose containing 1 mg/mL avidin (see text).

Depletion of Daudi Cells With Antibody 2H7 Followed by B-GAMIg

Very effective removal of Daudi cells from bone marrow was achieved when the cell mixture was treated successively with antibody 2H7 and B-GAMIg and then passed over a column containing avidin-Sepharose 6MB gel (Fig 2). More than two logs of Daudi cells were removed when dilutions of B-GAMIg ranging from 1:50 to 1:500 were used, and more than one log of Daudi cells could be eliminated with dilutions as high as 1:2,000 (Fig 2). Marrow recovery improved as the dilution was increased from 1:50 to 1:120 and then remained constant between dilutions of 1:160 and 1:8,000 (Fig 2). Increasing the concentration of antibody 2H7 beyond saturating levels (100 μg/mL) did not improve Daudi cell removal when B-GAMIg dilution of 1:50 was used

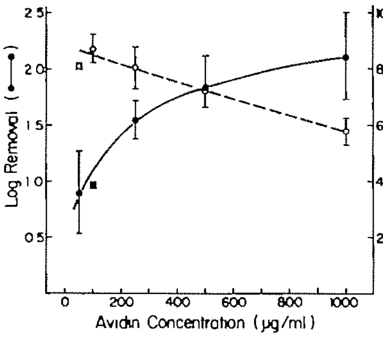

Fig 1. The effect of the concentration of avidin linked to Sepharose on the log removal of Daudi cells and percent recovery of bone marrow after treatment with biotinylated antibody 2H7. Fifteen million bone marrow cells containing 5% to 10% Daudi cells were treated with 100 μg/mL biotinylated antibody 2H7 and passed over a 3-mL column bed of Sepharose linked to the indicated amounts of avidin (see text). Each data point represents the results of two to four separate experiments.

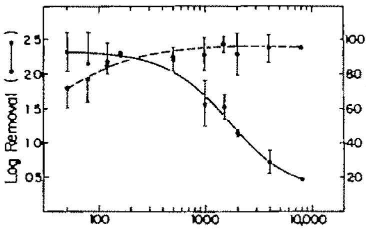

Fig 2. The effect of B-GAMIg dilution on log removal of Daudi cells and percent recovery of bone marrow after treatment with antibody 2H7. Fifteen million bone marrow cells containing 5% to 10% Daudi cells were treated successively with 200 μg/mL antibody 2H7 and the indicated dilutions of B-GAMIg and then passed over a 3-mL column bed of Sepharose containing 1.0 mg/mL avidin (see text). Each data point (except 1:160 and 1:8,000) represents the results of two to 11 separate experiments.

(2.37 ± 0.02 log removal at 100 μg/mL *v* 2.31 ± 0.28 log removal at 200 μg/mL.).

Control experiments demonstrated the necessity for both a monoclonal antibody reactive with the tumor cell and B-GAMIg (Table 2). Treatment of the bone marrow and Daudi cell mixture with antibody 2H7 and nonbiotinylated GAMIg or without a second antibody did not produce either removal of Daudi cells or loss of bone marrow. Using a monoclonal antibody nonreactive with Daudi cells and human bone marrow also had no effect on Daudi cell depletion. Daudi cells were not removed if treated cells were passed over a column constructed with 1 mg/mL albumin linked to Sepharose. Finally, treatment of a mixture of bone marrow and the Bp32 negative T cell line, Jurkat, with antibody 2H7 followed by B-GAMIg did not result in tumor cell removal.

Depletion of Daudi Cells With Antibody 2H7 Followed by B-F(ab')$_2$-GAMIg

Daudi cells were eliminated from bone marrow when treated successively with antibody 2H7 and B-F(ab')$_2$-GAMIg and then passed over avidin-Sepharose (Table 3). Very high concentrations of B-F(ab')$_2$-GAMIg were required for optional Daudi cell removal. Essentially no differences were observed in Daudi cell removal whether cells were treated with 100 or 200 μg/mL of antibody 2H7. Excellent recovery of bone marrow was observed even at a dilution of 1:10 B-F(ab')$_2$-GAMIg, but Daudi cell depletion was decreased in these experiments compared with results using B-GAMIg.

Table 2. Avidin-Biotin Immunoadsorption: Control Experiments

	No. of Tumor Cells/10^6 Marrow Cells			
Treatment	Pre-treatment	Post-treatment	log Removal	% Recovery
Antibody 2H7 alone*	7,200	7,400	0	100
Antibody 2H7 + GAMIg†	5,000	5,050	0	100
Nonreactive antibody (9E8) + B-GAMIg‡	5,630	5,750	0	88.5
Sham column§	4,650	5,580	0	85.9
Jurkat cells‖	4,850	6,550	0	81.8

Fifteen million bone marrow cells containing 5% to 10% Daudi (or Jurkat) cells were treated as indicated and passed over a 3-mL column bed of Sepharose (see text).

*Treatment with 200 μg/mL antibody 2H7 and passage over Sepharose containing 1 mg/mL avidin.

†Successive treatment with 200 μg/mL antibody 2H7 and 1 160 dilution of GAMIg (without biotin), followed by passage over Sepharose containing 1 mg/mL avidin.

‡Successive treatment with 200 μg/mL antibody 9E8, nonreactive with human marrow or Daudi cells, and 1:100 dilution of B-GAMIg, followed by passage over Sepharose containing 1 mg/mL avidin.

§Successive treatment with 200 μg/mL antibody 2H7 and 1:100 dilution of B-GAMIg, followed by passage over Sepharose containing 1 mg/mL albumin

‖Successive treatment of mixture of bone marrow and Jurkat cells, which do not express Bp32 antigen, with 200 μg/mL antibody 2H7 and 1:100 dilution of B-GAMIg, followed by passage over Sepharose containing 1 mg/mL avidin.

Table 3. Removal of Daudi Cells From Bone Marrow With Antibody 2H7 and B-F(ab')$_2$-GAMIg

2H7 Concentration (μg/mL)	B-GAMF (ab')$_2$ Dilution	log Removal	% Recovery
200	1.5	1 83	72.8
100	1:10	1.82 ± 0.13	92.3 ± 10.7
200	1:10	1.92 ± 0.06	88.3 ± 12.2
50	1:50	0.76	100
100	1:50	1.22	100
200	1:50	1.25	98.1
200	1:100	0.64	100

Fifteen million bone marrow cells containing 5% to 10% Daudi cells were treated successively with varying concentrations of antibody 2H7 and B-F(ab')$_2$-GAMIg and then passed over a 3-mL column bed of Sepharose with 1 mg/mL avidin (see text).

Depletion of Daudi Cells With Antibody DM1 Followed by B-GAMIg

Effective removal of Daudi cells and excellent recovery of bone marrow were observed when mixtures of bone marrow and Daudi cells were treated successively with antibody DM1 and B-GAMIg and then passed over an avidin-Sepharose 6MB column. Little variability was observed using antibody DM1 at concentrations from 10 to 30 μg/mL (and a constant 1:100 dilution of B-GAMIg), with approximately 2.5 logs of Daudi cells removed and 80% to 85% recovery of bone marrow (10 μg/mL – 2.53 ± 0.28 log removal, 84.0 ± 13.8% recovery; 20 μg/mL – 2.45 ± 0.09 log removal, 79.5 ± 6.4% recovery; 30 μg/mL – 2.61 ± 0.04 log removal, 85.4 ± 0.3% recovery). Identical results were observed when mixtures of Daudi cells and bone marrow cells were treated in the presence or absence of 0.1% sodium azide, suggesting that antigenic modulation does not affect Daudi cell removal (data not shown).

Experiments were also performed to study the efficiency of Daudi cell removal when either 10 or 30 μg/mL of antibody DM1 and various dilutions of B-GAMIg were used (Fig 3). Nearly three logs of Daudi cells were eliminated when the bone marrow and Daudi cell mixture was treated with 30 μg/mL of antibody DM1 followed by 1:50 dilution of B-GAMIg (Fig 3B). Similar levels of depletion were observed when cells were first treated with 10 or 30 μg/mL of antibody DM1 and then with B-GAMIg at a dilution of 1:100. Daudi cells were less efficiently removed when the dilution of B-GAMIg was increased above 1:100 after treatment with antibody DM1 at 10 μg/mL (Fig 3A). In contrast, more than two logs of Daudi cells were eliminated using B-GAMIg at a dilution of 1:200 with cells treated with 30 μg/mL of antibody DM1 (Fig 3B). Marrow recovery was remarkably similar when cells were treated with either 10 μg/mL or 30 μg/mL of antibody DM1. Compared to results with antibody 2H7, Daudi cell depletion after treatment with antibody DM1 appeared to be slightly better at the lower dilutions of B-GAMIg. However, removal of Daudi cells with antibody 2H7 was more than two logs over a wide concentration range of B-GAMIg, while removal of Daudi cells with antibody DM1 was less than two logs when B-GAMIg dilutions greater than 1:200 were used.

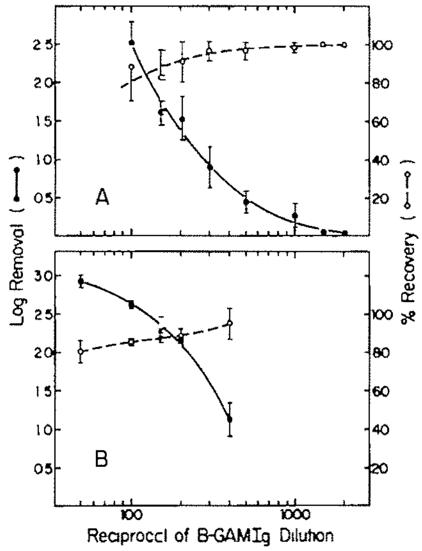

Fig 3. The effect of B-GAMIg dilution on log removal of Daudi cells and percent recovery of bone marrow after treatment with 10 μg/mL (A) or 30 μg/mL antibody DM1 (B). Fifteen million bone marrow cells containing 5% to 10% Daudi cells were treated successively with 10 μg/mL or 30 μg/mL antibody DM1 and B-GAMIg at the indicated dilutions and then passed over a 3-mL column bed of Sepharose containing 1.0 mg/mL avidin (see text). Each data point (except 1:1,500 and 1:2,000) represents the results of two to four separate experiments.

Passage of the treated mixture of bone marrow and Daudi cells over columns containing increased amounts of avidin on Sepharose 6MB resulted in more effective depletion of Daudi cells (Table 4). Recovery of bone marrow was decreased when the amount of avidin on the gel was increased. When the cells were treated with a higher dilution of B-GAMIg (1:200), bone marrow recovery was improved to 91.4 ± 10.7% after passage over a 2-mg/mL avidin gel, but depletion of Daudi cells was decreased (2.49 ± 0.02 log removal) compared to results when a 1:100 dilution of this antiserum and the 2-mg/mL avidin gel were used (3.05 ± 0.24 log removal and 69.6 ± 10.6% recovery).

Control experiment again demonstrated the requirement for both a monoclonal antibody reactive with the tumor cell and B-GAMIg (data not shown). Treatment of the mixture of bone marrow and Daudi cells with antibody DM1 and nonbiotinylated GAMIg or without a second antibody did not produce Daudi cell removal or loss of bone marrow. Successive treatment with antibody DM1 and B-GAMIg and then passage over Sepharose containing 1 mg/mL albumin did not remove Daudi cells. Successive treatment of a mixture of bone marrow containing 5% Jurkat cells with antibody DM1 and B-GAMIg and then passage over avidin-Sepharose did not eliminate Jurkat cells.

Recovery of Hematopoietic Progenitors

The growth of hematopoietic progenitors (CFU-C) was assessed in two separate experiments. In the pretreatment group ("Pre"), bone marrow mononuclear cells were obtained by Ficoll-Hypaque density centrifugation, mixed with 10% irradiated (1,500 rad) Daudi cells, after which the assay for CFU-C was performed. In the posttreatment group ("Post"), the bone marrow mononuclear cells and irradiated Daudi cells were successively treated with 10 μg/mL DM1 and 1:100 dilution of B-GAMIg and then passed over a sterile 3-mL column bed of Sepharose containing 1 mg/mL avidin, after which plating for CFU-C was performed. Preliminary experiments showed that irradiated Daudi cells did not grow under the conditions employed for the growth of hematopoietic precursors. Preservation of CFU-C was noted in both experiments (Table 5). Furthermore, nearly quantitative recovery of these hematopoietic progenitors was observed, averaging 96.2 ± 3.3% and 110.7 ± 12.0% in the two experiments. Control experiments showed that the growth of CFU-C was not affected by irradiated Daudi cells (data not shown). As an additional control, experiment 3 demonstrated that column treatment alone also had no effect on growth or recovery of CFU-C.

Table 4. Effect of Avidin Concentration on Removal of Daudi Cells and Recovery of Bone Marrow

Avidin Concentration (mg/mL)	log Removal	% Recovery
0.5	2.41 ± 22	85.5 ± 6.6
0.75	2.54 ± .38	80.2 ± .07
1.0	2.53 ± .28	84.0 ± 13.8
1.5	2 76 ± .26	67.9 ± 8 7
2.0	3 05 ± .24	69.6 ± 10.6

Fifteen million bone marrow cells containing 5% to 10% Daudi cells treated successively with 10 μg/mL antibody DM1 and 1:100 dilution of B-GAMIg and then passed over a 3-mL column bed of Sepharose containing the indicated concentrations of avidin (see text). Each avidin concentration was tested in two to four separate experiments

DISCUSSION

The high binding affinity between avidin and biotin has encouraged their application to a variety of fields of biology and protein chemistry, including the isolation of biotin-derivatized materials by affinity chromatography, affinity labeling using biotinylated antibodies, and the development of various immunoassays. Investigators have recently demonstrated that this complex can be used for selective binding of cells as well. Jasiewicz et al[21] first showed that biotin-labeled thymocytes could be bound to nylon mesh containing immobilized avidin. Wormmeester et al[22] reported using biotinylated antibodies and avidin-coated sheep erythrocytes to separate various murine lymphoid populations. Basch et al[23] reported that Thy-1.2-positive cells could be attached to either plastic dishes or polyacrylamide beads containing avidin after treatment with biotinylated anti-Thy-1.2 antibody. We have now used biotinylated monoclonal antibodies and avidin bound to Sepharose to eliminate Daudi tumor cells from human bone marrow. We have also developed an indirect approach of treating a mixture of bone marrow and Daudi cells successively with nonbiotinylated murine monoclonal antibodies and biotinylated goat antimouse antiserum followed by passage over avidin-Sepharose that successfully removes Daudi cells from bone marrow.

Chromium-51 release assays and immunofluorescence

Table 5. Assessment of Effects on Hematopoietic Progenitors (CFU-C)

Experiment	No. of CFU-C/10^5 Marrow Cells*		% Total Recovery†
	Pre	Post	
1 (treated cells)	53.3 ± 10.4	79.3 ± 14.6	110.7 ± 12.0
2 (treated cells)	35.3 ± 2.9	42.3 ± 4.0	98.2 ± 3.3
3 (untreated cells)	47.0 ± 4.2	49.6 ± 9.1	108.7 ± 6.7

In experiments 1 and 2, 15 × 10^6 bone marrow cells containing 10% irradiated Daudi cells were incubated successively with antibody DM1 and B-GAMIg and then passed over a 3-mL column bed of Sepharose containing 1 mg/mL avidin (see text). In experiment 3, cells were passed over an avidin-Sepharose column without prior antibody DM1 or B-GAMIg treatment.

*The value shown represents the mean of samples that were plated and counted in triplicate. "Pre" indicates the number of CFU-C grown before treatment, while "Post" shows the number of CFU-C after treatment.

†Percent recovery of total CFU-C after treatment, calculated as follows.

$$100 \times \frac{(\text{Post cell number}) \times (\text{Post CFU-C})}{(\text{Pre cell number}) \times (\text{Pre CFU-C})}$$

techniques are capable of detecting on the order of 0.01% and 0.1% residual tumor cells contaminating bone marrow.[13] Clonogenic assays that have been recently described cannot be used with many tumor cell lines and require several days to obtain results.[24] Our assay is similar to that described by Reynolds et al[25] in using a fluorescent dye to label tumor cells, which can be readily detected and rapidly quantitated with an inverted fluorescence microscope. This assay can detect one FITC-labeled tumor cell in 1 × 10^6 bone marrow mononuclear cells. We have used the FITC dye, which Butcher and Weissman previously showed produces bright intracellular labeling of viable lymphoid cells that remains for days without affecting the expression of cell surface antigens.[18] Labeling of tumor cells with FITC can be completed in less than one hour, and significant leakage of FITC from labeled cells that can potentially stain adjacent bone marrow does not occur.

By incubating a mixture of bone marrow and 5% to 10% Daudi cells with the biotinylated monoclonal antibody 2H7, which recognizes the Bp32 (CD20) antigen present on Daudi cells, we were able to remove up to two logs of Daudi cells from bone marrow. The increased concentrations of avidin on the gel required to achieve highly effective Daudi cell depletion were associated with decreased recovery of unbound cells. We could not improve recovery by blocking Fc receptors or potential binding sites for biotin on cells with unconjugated, nonreactive antibody or free biotin. Investigators have postulated that biotinylation increases the hydrophobicity of the antibody molecule and that this causes nonspecific binding of biotinylated antibodies on cell surfaces.[26] We thus attempted to decrease the nonspecific loss of bone marrow by decreasing the biotin-antibody conjugation ratio. However, improved recovery of bone marrow was accompanied by decreased removal of Daudi cells.

As much as three logs of Daudi cells could be removed from bone marrow with the use of an indirect "sandwich" technique. The use of a biotinylated goat antimouse antiserum eliminates the need to prepare a biotin conjugate of each individual monoclonal antibody. In comparison to treatment with biotinylated antibody 2H7, the indirect technique also yielded a significant improvement in the recovery of bone marrow. Whereas recovery was reduced below 60% at concentrations of biotinylated antibody 2H7 required to achieve more than two logs of Daudi cell elimination, marrow recoveries up to 90% could be achieved by using dilutions of B-GAMIg that gave a similar degree of Daudi cell depletion. Bone marrow recovery also decreased as the concentration of B-GAMIg was increased. Similar levels of Daudi cell depletion were achieved with either antibody 2H7 or antibody DM1 using B-GAMIg. The slightly improved recovery noted with antibody DM1 compared to antibody 2H7 observed using lower dilutions of B-GAMIg is partly explained by the fact that approximately 4% to 6% of normal bone marrow cells express Bp32, while only 1% to 2% of these cells express surface IgM (data not shown).

Depletion was slightly decreased when attempts were made to remove Daudi cells with a combination of antibody 2H7 and B-F(ab′)$_2$-GAMIg, although marrow recovery was improved. Dilutions of this biotinylated antiserum as low as 1:10 (70 μg/mL) could not remove more than two logs of Daudi cells. It is possible that differences in the affinity of GAMIg and the F(ab′)$_2$ molecule for mouse IgG could explain the findings. A decreased number of biotin conjugation sites on the smaller F(ab′)$_2$ fragment could also explain the decrease in both specific and nonspecific removal of cells.

Incomplete removal of Daudi cells from bone marrow appears to be primarily due to decreased antigen density on some cells We have found that the Daudi cells remaining after column treatment do not express detectable levels of antigen by immunofluorescence staining (data not shown). These tumor cells may represent a truly antigen-negative variant subpopulation or may express amounts of antigen that are below the sensitivity of immunofluorescence methods. Heterogeneity of antigenic expression has also been observed with other tumor cell lines.[27] Cloning of tumor cell lines may produce populations of cells that have more uniform expression of selected antigens.

We have initiated studies directed at scaling up the procedure to treat the number of cells needed for clinical application of this technique to BMT. Preliminary experiments have demonstrated that up to 200 × 10^6 bone marrow containing 10% Daudi cells successively treated with antibody DM1 and B-GAMIg can be passed over a 3-mL column bed of avidin-Sepharose with Daudi cell removal and marrow recovery similar to the results observed with 15 × 10^6 cells (data not shown). Based on these preliminary data, we estimate that a 100 to 125-mL column bed of avidin-Sepharose could accommodate the 5 to 10 × 10^9 bone marrow mononuclear cells required for BMT.

A comparison of the avidin-biotin system described here and other in vitro methods (including antibody and complement, antibody-ricin conjugates, and immunomagnetic beads) designed to remove tumor cells from bone marrow is currently being carried out. The column technique has the advantages of being easily performed in a relatively short period of time without significant loss of bone marrow cells or toxicity to hematopoietic precursors. Reliable and consistent results can readily be achieved. This technique may also prove useful as a means of removing T cells from bone marrow for the prevention of GVHD.

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Abnormalities of Chromosome 7q and Tac Expression in T Cell Leukemias

By Vasantha Brito-Babapulle, Estela Matutes, Leonor Parreira, and Daniel Catovsky

Clonal chromosome abnormalities were demonstrated in 13 cases of T cell leukemia with a mature membrane phenotype. The cases comprised two adult T cell lymphoma leukemia (ATLL), five Sézary syndrome (SS), three T prolymphocytic leukemia (T-PLL), and three T chronic lymphocytic leukemia (T-CLL). The presence of clonal chromosome abnormalities in the three cases of T-CLL supports the view that although clinically benign, this disorder probably represents a neoplastic process.

Cells from six of 11 patients were tested and found to be reactive with monoclonal antibody (McAb) anti-Tac in the absence of mitogens (two ATLL, two SS, and two T-PLL). Only the two ATLL patients were serologically positive for the human T cell leukemia virus I (HTLV-I), and their cells showed a higher anti-Tac reactivity. Chromosome 7 abnormalities were observed in seven cases (two ATLL, three SS, two T-PLL), and a strong correlation was seen between the presence of a 7q abnormality and the expression of the Tac antigen (T cell growth factor receptor). These findings are discussed in relation to the localization of T cell receptor genes on chromosome 7 and possible mechanisms for the activation of the Tac gene.

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PREVIOUS CYTOGENETIC studies on T cell leukemia have not shown any obvious relationship between chromosome abnormalities and the expression of factors that may be associated with T cell proliferation. Lectin-stimulated proliferation of normal mature T cells is associated with the production of T cell growth factor (TCGF) and the expression of its receptor (Tac antigen).[1] T cells from human T cell leukemia virus (HTLV-I)–induced malignancies are known to express the Tac antigen in the absence of lectin stimulation, either in the resting phase or after culture.[2,3] Cells from other T leukemias rarely express the Tac antigen in the absence of stimulation.[2,3] It is also becoming increasingly clear that chromosomal regions bearing the T cell receptor genes may be significant in the generation of chromosome abnormalities in many T cell disorders.[4,5]

We have carried out cytogenetic studies and demonstrated clonal chromosome abnormalities in 13 cases of T cell leukemia resulting from the expansion of populations of neoplastic T lymphocytes with a mature (postthymic) phenotype. Cells from 11 of these patients were tested for the membrane expression of the Tac antigen with a specific monoclonal antibody (McAb).[6] A striking correlation was observed between abnormalities of 7q and expression of Tac.

MATERIALS AND METHODS

Patients. Cytogenetic studies and membrane marker analysis were carried out on mononuclear cells from peripheral blood samples of 13 patients with T cell leukemia. The T cell malignancies were classified according to clinical, morphological, and immunologic criteria. HTLV-I-induced malignancies were demonstrated by the presence of serum antibodies (kindly determined by Dr A. Dalgleish) and by the reactivity of the leukemic cells with McAb against the HTLV-I core proteins p19 and p24 (gift from Dr B.F. Haynes). Two patients (Nos. 1 and 2) were black and had HTLV-I-positive adult T cell lymphoma leukemia (ATLL), while all the others were HTLV-I-negative. Clinical details of the two ATLL patients have been reported elsewhere.[7,8] Five patients (Nos. 3 to 7) had Sézary syndrome (SS), which was characterized by moderate lymphadenopathy, erythroderma, and the presence of typical cerebriform lymphocytes in the peripheral blood. Three patients (Nos. 8 to 10) had T prolymphocytic leukemia (T-PLL) with marked hepatosplenomegaly, high WBC count, and bone marrow infiltration by typical prolymphocytes. Three patients (Nos. 11 to 13) had T chronic lymphocytic leukemia (T-CLL). One of them was asymptomatic, and the other had neutropenia, infections, and splenomegaly. Two of the three had a past history of rheumatoid arthritis (RA) and had been splenectomized earlier. All three T-CLL patients had mature-looking granular lymphocytes in the peripheral blood.

Membrane marker analysis This was carried out on mononuclear cells in suspension by indirect immunofluorescence. The membrane phenotype of the T cell leukemias was characterized by E rosettes and a panel of McAb: UCHT1 (T3;CD3),[9] OKT4/Leu-3 (T4;CD4),[10] OKT8 (T8;CD8),[11] OKT6 (T6;CD6),[12] and OKT17 (T17).[13] Terminal transferase (TdT)[14] was assessed on cytocentrifuge slides fixed in acetone-chloroform by an indirect immunoperoxidase method. The presence of receptors for TCGF was investigated with the McAb anti-Tac[6] in fresh and short-term cultured (72 to 172 hours) cells. In four cases, the immunogold method (IGM)[15] was applied at electron-microscopic level in order to characterize the morphology of the cells reactive with anti-Tac.

Cytogenetic studies. Mononuclear cells were seeded at a concentration of 1.5 to 2.0 × 10^6 cells/mL in medium RPMI-1640 supplemented with fetal calf serum (20%). The cells were cultured in the presence of phytohemagglutinin (PHA) 0.2 μg/mL and TCGF (1 mL/10 mL culture) for 5 to 10 days. Cells were harvested after incubation with 0.05 μg/mL colcemid (Gibco) for one hour, followed by hypotonic treatment and fixed in one part glacial acetic acid to three parts methanol. Giemsa banding was carried out on air-dried slides by routine methods

RESULTS

Membrane markers and reactivity with anti-tac. The cells in all cases had a mature (postthymic) phenotype: E^+, TdT^-, $T6^-$, $T3^+$, and $T17^+$. In addition, these cells were $T4^+$ or $T8^+$, except in patient 2 (ATLL), whose cells were unreactive with the McAb against the two major lymphocyte subsets (Table 1).

Fresh and/or unstimulated lymphoid cells from six cases (two ATLL, two SS, and two T-PLL) of 11 cases tested showed reactivity with the McAb anti-Tac (8% to 59% of positive cells).

The IgM method demonstrated Tac positivity in the

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Table 1. Correlation Between Abnormalities of Chromosome 7 and Expression of IL 2 Receptors*

Disease	Case No.	Phenotype	Anti-Tac† (%)	Chromosome 7q Abnormality
ATLL	1	T4+	52	Present
	2	T4–,T8–	10	Present
SS	3	T4+	0	Absent
	4		—	Present
	5		—	Absent
	6		20	Present
	7		8	Present
T-PLL	8	T4+	0	Absent
	9	T8+	59	Present
	10	T4+	11	Present
T-CLL	11	T4+	0	Absent
	12	T8+	0	Absent
	13	T8+	0	Absent

*Demonstrated by reactivity with McAb anti-Tac without mitogenic stimulation.

†The percentage of anti-Tac-positive leukemic cells was possibly higher in patients 2, 6, and 7 since the proportion of neoplastic cells in these samples ranged from 20% to 30% of the mononuclear fraction.

leukemic cells. ATLL cells displayed a higher degree of reactivity with anti-Tac when compared with the cells from the other T cell leukemias as demonstrated by the number of colloidal gold particles attached to the cell membrane (Fig 1).

Cytogenetics. Clonal chromosome abnormalities were observed in 13 cases of T cell leukemia and are summarized in Table 2. Case 1 has been described in detail elsewhere.[7] Seven cases had abnormalities of chromosome 7 (two ATLL, three SS, two T-PLL) (Fig 3). Rearrangements involving breakpoint 7p14 were present in three patients (two ATLL, one SS), and abnormalities with breakpoint 7q35–36 were observed in four others (two SS, two T-PLL). Trisomy or partial trisomy for 7q was observed in six (two ATLL, two SS, two T-PLL).

Correlation between chromosome 7 abnormalities and anti-Tac reactivity. The cells from 11 patients were tested for anti-Tac reactivity (Table 1). Of these, six showed anti-Tac reactivity (cases 1, 2, 6, 7, 9, and 10). All six had abnormalities of chromosome 7q. The Tac-negative patients had no chromosome 7 abnormalities.

Case 3 (SS) with i(7q) and case 4 (SS) were not tested with anti-Tac.

DISCUSSION

We have described clonal chromosome abnormalities in 13 cases of T cell leukemia, and the presence of chromosome 7 rearrangements were found to correlate with Tac expression in the leukemic cells.

The demonstration of clonal abnormalities in the three cases of T-CLL in our study is of interest. Although it has been recognized that T-CLL constitutes a distinct clinical entity, its chronic course and difficulty in demonstrating clonality have led to the use of the uncommitted term *chronic T cell lymphocytosis*.[16] We are aware of only four cases where clonal chromosome abnormalities have been demonstrated in this disease.[17–19] The findings in this study, together with the possibility of demonstrating rearrangements of the T cell receptor genes,[20] strengthens the concept that T-CLL is clonal in nature and probably represents a neoplastic process.

One patient in this study had an inversion involving 14q11 and 14q32. Abnormalities of chromosome 14 with q11-13 as breakpoint have been reported in T-ALL,[21] T-PLL,[22] SS,[23] chronic T cell leukemias,[24,25] and T cell leukemias in patients with ataxia telangiectasia (AT).[24,26] The rearrangements are usually inversions or tandem translocations involving 14q11 and 14q32 and translocations between other autosomes and 14q11-13. In this respect the recent localization to 14q11 of the T cell receptor α-chain gene, which rearranges during T cell differentiation, is significant,[5] and a possible oncogene has been postulated on 14q32 proximal to the IgH locus.[5] The rearrangements are thought to act in a manner analogous to translocations in Burkitt's lymphoma, where the immunoglobulin genes and the *c-myc* oncogene are brought adjacent to each other.[5,27]

The presence of a chromosome 7 abnormality in seven of

Table 2. Chromosome Abnormalities in 13 Cases of T Cell Leukemia

Disease	Case No.	Chromosome No.	No. of Abnormal Cells	Significant Clonal Abnormalities*
ATLL	1	50XXY	17/35	+3,+del 7(p14),1q+,6q–(q21),t(11;13)(q14;q22–24),i(17q)
	2	47XY	3/6	+del 7(p14)
SS	3	46XXY	10/10	–15,t(1;17;?),2p+(p23)
	4	88XXXX	15/15	2p–(p14),2p–(p14),6q–(q21),6q–(q21),i(7q)i(7q)
	5	44XX/88XX	20/20	2p–(p14–15)/2p–(p14–15),2p–(p14–15)
	6	46XY	3/8	6q–(q23), t(7;7;7)(p14;q11–35;q11–32)
	7	46XY	7/10	6q–(q21),t(2;7)(p11–12;q35–36),M
T-PLL	8	46XY	5/8	t(8;8)(p12;q11),12q+,inv.14(q11–32)†
	9	46Xq+Y	5/5	inv.dup(7)(q21–q35–36),t(7;15)(q34–35; q13),i(8q),dup.X(q12-qter)
	10	42XO	3/6	1p+,7p+,6q–,+7p+q+(q35)
T-CLL	11	46XX	4/9	t(4;17)(p15–16;q23)
	12	46XO	15/15	6q–(q21),t(11;15)(q13;q22–24)
	13	46XX	3/8	int.del (14)(q22–24)

*Chromosome 7 abnormalities are underlined.

†See Fig 2.

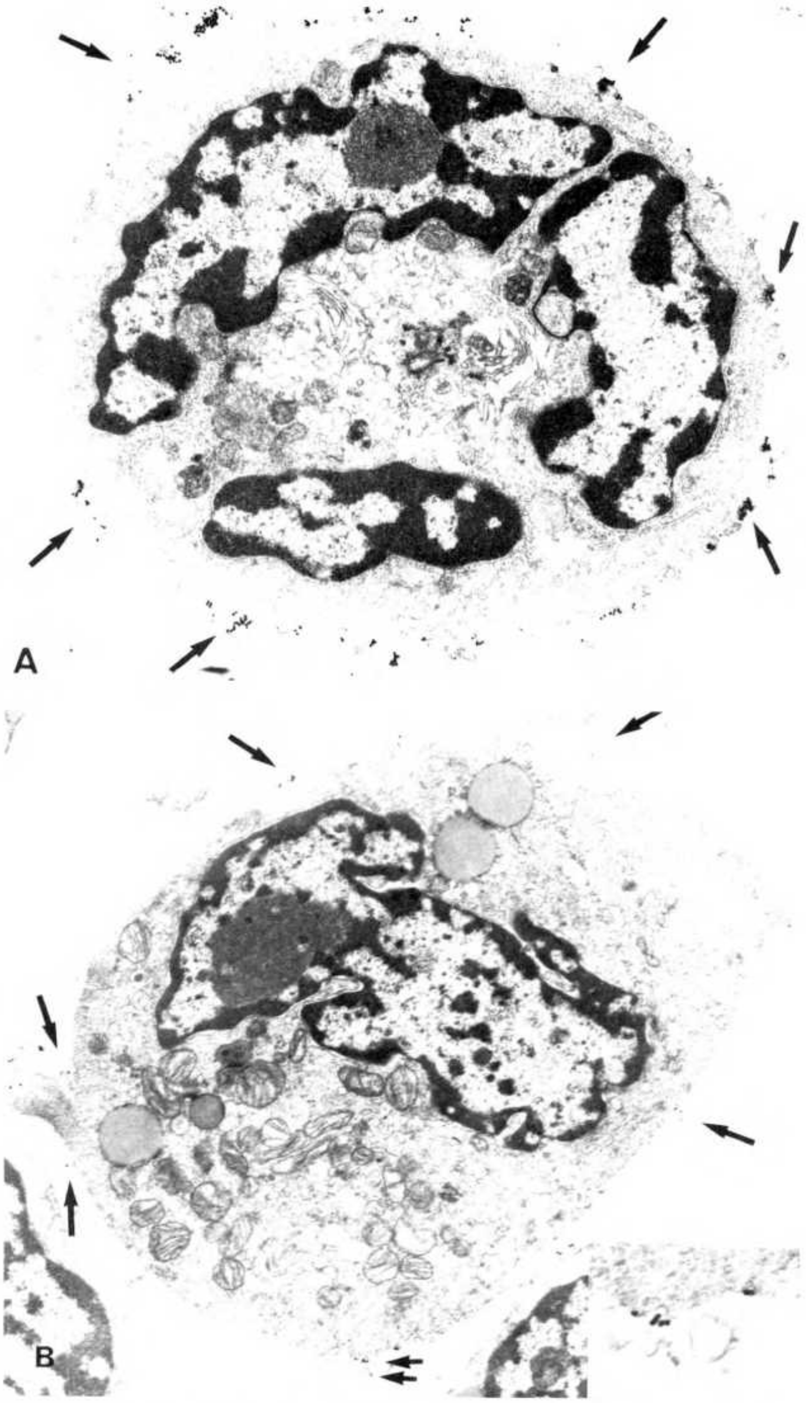

Fig 1. Anti-Tac reactivity is shown by colloidal gold particles attached to the cell membrane. (A), ATLL-lymphoid cell (case 1); (B) Sézary cell (case 6); (C) T prolymphocyte (case 9); (D) large granular lymphocyte characteristic of T-CLL (case 12). The strongest reactivity is seen in ATLL cells whereas T-CLL lymphocytes are negative. Insets in B and C are higher magnifications of the regions indicated by the double arrows (uranyl acetate and Reynold's lead citrate stain).

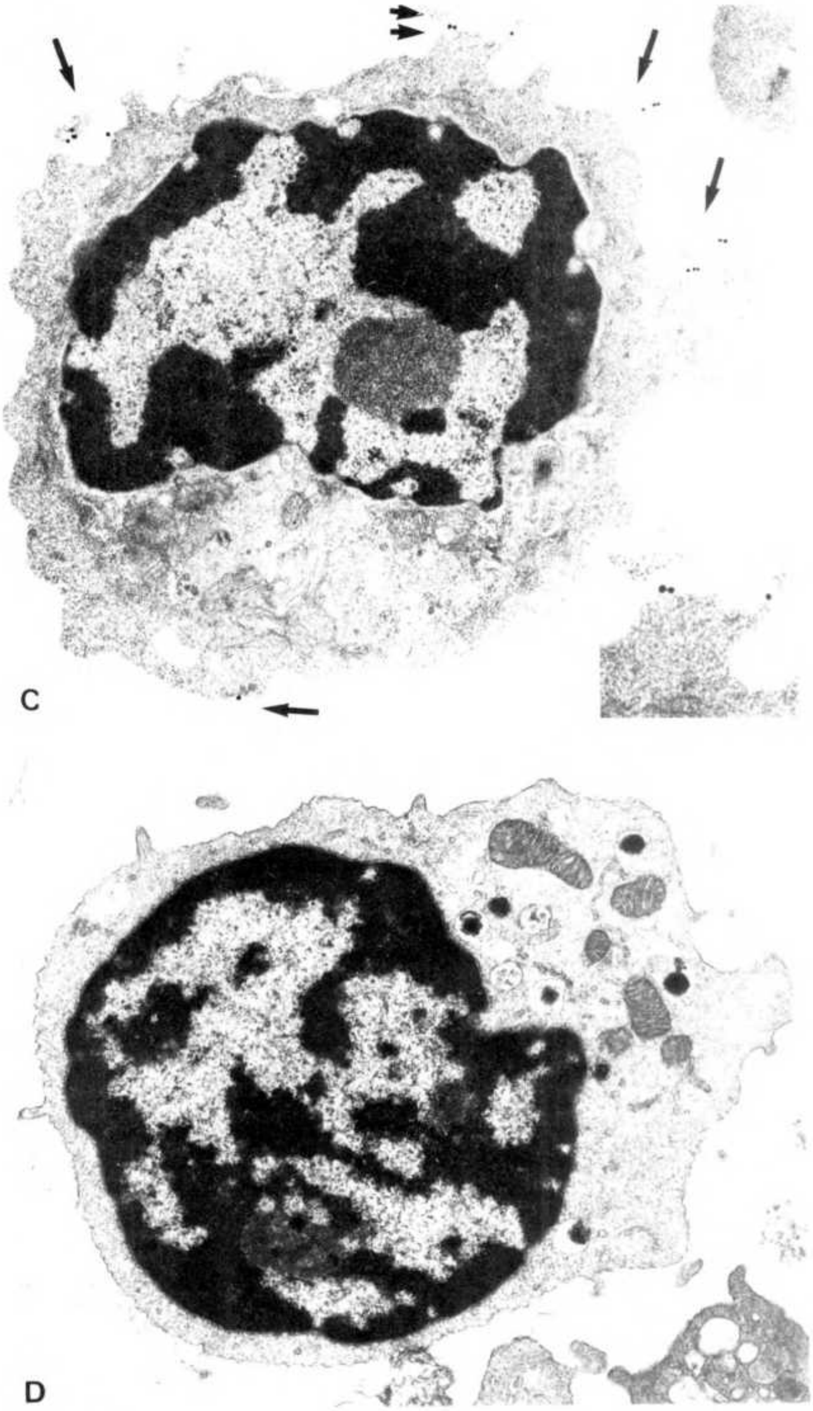

Fig 1 (Cont'd).

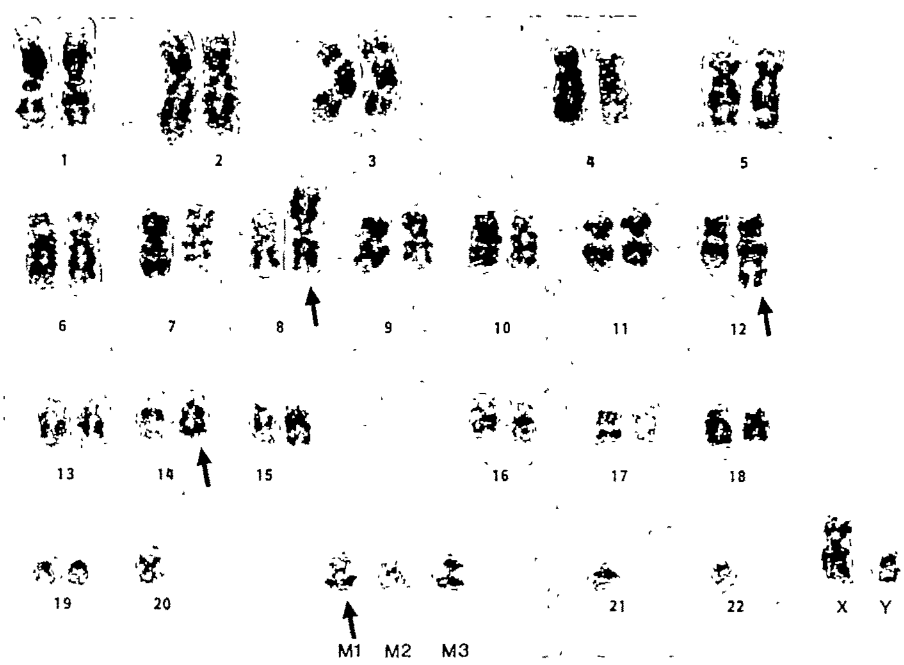

Fig 2. Karyotype of a cell from case 8 (T-PLL): 46XY, −20,−21,−22,2q−,6p+,t(8;8)(p12;q11),12q+inv14(q11-q32), M_1,M_2,M_3. Arrows indicate clonal chromosome abnormalities.

13 cases in our study is of particular interest. Based on the frequent rearrangements between 7p14, 7q35, 14q11, and 14q32 in T cells of patients with AT, Fiorelli et al[28] have speculated that T cell receptor genes may be present on 7p14 and 7q35. In fact, the T cell receptor β-chain gene has been mapped to 7q35,[29] while the γ chain gene has been assigned to 7p (T.H. Rabbitts, personal communication). The involvement of 7p14 and 7q35 in six of seven cases with chromosome 7 abnormalities in our study is significant. The most important and intriguing finding in the present study has been the correlation observed between abnormality of 7q and the expression of the Tac antigen in the absence of mitogenic stimulation. Of the six cases showing anti-Tac reactivity, five had trisomy or partial trisomy for 7q. Trisomy for 7q has been described in one third of patients with ATLL,[30] especially in cases with an acute clinical course.[31] Since marked anti-Tac reactivity is observed in leukemic cells of the majority of ATLL patients,[2,3] it would appear that infection with HTLV-I induces Tac expression independently of chromosome 7q abnormalities. The exact mechanism and role of Tac expression associated with HTLV-I are still not clear,[32] since most T cell lines derived from ATLL patients grow in the absence of exogenous TCGF and have no detectable messenger RNA for endogenous TCGF production.[33] This suggests that HTLV-I infection induces cell proliferation in these T-cell lines by a mechanism that bypasses TCGF-Tac binding.

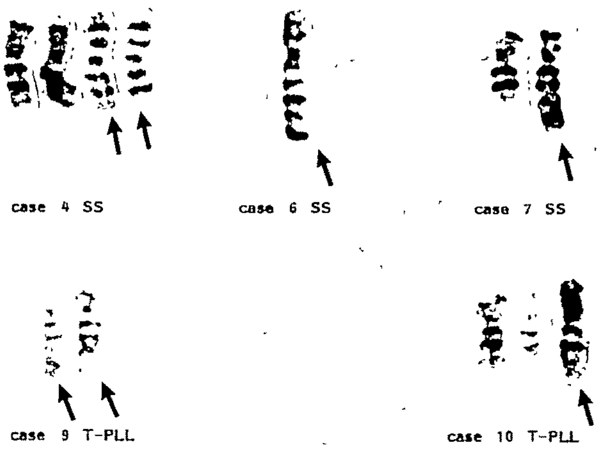

Fig 3. Chromosome 7 abnormalities in three cases of SS and two cases of T-PLL. Case 4: i(7q)(qter→cent→qter) resulting in trisomy 7q. Case 6: t(7;7;7) (qter cent→p14:q11→q35:q11−q32) resulting in partial trisomy for 7q. Case 7: t(2;7)(2pter→2p11-12:7q35-36→7pter). Case 8: inv.dup.(7)(pter→q35-36:q35-36—q21) resulting in partial trisomy for 7q. t(7;15)(7pter→q34-35:15q13→15qter). Case 10: Addition of 7p+q+ resulting in trisomy for chromosome 7. Breakpoints on p and q are 22 and 35, respectively.

The structural gene for the Tac antigen has been mapped to chromosome 10p14-15.[34] Therefore, the association between 7q abnormalities and Tac expression is unlikely to result from an alteration of the Tac gene. Since the gene for the T cell receptor β chain is mapped to 7q35,[29] activation of this antigen receptor may result indirectly in Tac expression. Thus it is possible to speculate that 7q abnormalities produce an activated phenotype by alteration of the T cell receptor locus. Alternatively, there may be other genes on 7q that are deregulated and amplified by the chromosome abnormalities, leading to the activation of the Tac gene by a transregulatory mechanism.

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Effect of Leukocyte Antibodies and HLA Matching on the Intravascular Recovery, Survival, and Tissue Localization of 111-Indium Granulocytes

By Jeffrey McCullough, Mary Clay, David Hurd, Karen Richards, Carl Ludvigsen, and Lee Forstrom

The effect of leukocyte antibodies detected under different conditions on the fate in vivo of granulocytes was studied using 111-indium-labeled granulocytes. Sera from patients were tested by granulocyte agglutination (GA), granulocytotoxicity (GC), granulocyte immunofluorescence (GIF), lymphocytotoxicity (LC), and antibody-dependent lymphocyte-mediated granulocytotoxicity. Granulocytes from donors to be studied were labeled with 111-indium and injected. Then the intravascular recovery and survival or tissue localization was determined in 93 studies. Antibodies detected by granulocyte agglutination were associated with a significant reduction in recovery (6.7% *v* 30.8% in controls; *P* < .001) and t½ (0.3 hours *v* 5.6 hours in controls; *P* = .002). When all possible combinations of serum reactivity were considered, reactivity in the GA plus GIF assays had the best correlation with decreased recovery (R^2 = .49; *P* < .001) and t½ (R^2 = .73; *P* < .001). When the relationship between the strength of antibody reactivity and the recovery and t½ were analyzed, the best relationship was between the combination of LC and GIF with recovery (R^2 = .62; *P* = .001). Because of the general availability of the HLA (LC) testing, the role of LC reactivity was investigated in other ways. There was a strong relationship between sera highly reactive by LC and those reactive by GIF. These highly reactive sera were also associated with reduced recovery and t½. The influence of specific HLA antigen mismatches was also studied. When donor and recipient were mismatched for the HLA-A2, B8, or BW44 antigens, there was a significant reduction in either recovery, t½, or both. Tissue localization was studied by body scans in patients with and without known sites of inflammation. Antibodies detected by a combination of GA and GIF caused abnormal pulmonary sequestration of granulocytes (three cases) and failure of granulocytes to localize at known sites of inflammation (three cases). HLA (LC) antibodies did not alter tissue localization despite the presence of the corresponding HLA antigens on granulocytes. It appears that GA, GIF, or a combination of these tests is the most effective predictor of altered in vivo fate of granulocytes. However, sera highly reactive by LC and GIF probably define a group of highly immunized patients in whom granulocyte recovery and t½ are also reduced. Mismatching for certain HLA antigens is also associated with reduced granulocyte recovery and survival. At present, GA, with or without the immunofluorescence assay, is the most effective predictor of altered in vivo granulocyte activity. LC, if interpreted in a specific manner, may also define a group of highly immunized patients in whom in vivo granulocyte activity is altered.

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LEUKOCYTE ALLOANTIBODIES play a role in febrile and pulmonary transfusion reactions, isoimmune neonatal neutropenia (INN), response to granulocyte transfusions, drug-related neutropenia, and autoimmune neutropenia.[1] Many methods are available to detect leukocyte antibodies; however, the relationship of these tests to immune destruction of granulocytes, and thus to these different clinical situations, is not established.[2–6] Because most of the assays are complex, there have been few studies comparing the value of different antibody methods in detecting clinical problems. For instance, in different reports, autoimmune neutropenia has been associated with antibodies detected by agglutination, cytotoxicity, immunofluorescence, opsonization, antibody-dependent cell-mediated cytotoxicity, and staphylococcal protein A.[1,6] Immune neutropenia secondary to drugs may be caused by granulocyte agglutinins, opsonins, or cytotoxins[1,7] and secondary to Felty's syndrome, by antibodies detected by antibody-dependent lymphocyte-mediated granulocytotoxicity.[8] Transfusion reactions are associated with leukoagglutinins and lymphocytotoxins (HLA antibodies), while antibodies detected by both granulocyte agglutination and immunofluorescence have been found in INN. A poor response to granulocyte transfusions has been attributed to different antibodies, including leukoagglutinins, lymphocytotoxins,[9–11] and those detected by immunofluorescence,[12,13] although others[14] have reported no effect of granulocyte agglutinins, granulocytotoxins, or lymphocytotoxins on granulocyte intravascular recovery

Until recently, there has not been a method available to study the in vivo fate of a single injection of a granulocyte suspension so that the effect of antibodies present at the time could be determined. The isotope ^{111}indium is an efficient granulocyte label, does not elute from the cell, and emits photons in high abundance, making it ideal for external body imaging.[15] Thus ^{111}indium granulocytes can be used to determine both the intravascular kinetics and the extravascular localization of granulocytes.[16,17] We have used ^{111}indium granulocytes to study the effect of leukocyte antibodies detected in five assays on the in vivo fate of granulocytes.

This report is an extension of our previous work.[17] Thirty-five new studies were done using all five leukocyte antibody methods, and two additional antibody methods were applied to the 53 studies previously reported.

MATERIALS AND METHODS

This study was approved by the University of Minnesota's Committee of the Use of Human Subjects in Research. All participants gave informed consent. Subjects in this study were either patients who were receiving platelet or granulocyte transfusions or those for whom an indium scan had been requested for diagnostic purposes

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because localized infection was suspected. On the morning of the indium study, blood was obtained from the patients and normal donors. A suspension of donor granulocytes was prepared, labeled with ^{111}indium, and injected and the intravascular kinetics and organ localization studies done during the subsequent 24 hours. A crossmatch between the recipient's (patient's) serum and donor's granulocytes and lymphocytes was done using five leukocyte antibody methods. Sometimes it was not possible to do all five crossmatches on the day of the indium study. In those cases, the patient's serum from that day was stored at −70 °C and used for crossmatching later with granulocytes or lymphocytes freshly obtained from the original donor. The granulocyte donors met all of the criteria of the American Association of Blood Banks for whole blood donation and were hepatitis B_s antigen negative. All of the methods used in this study have been reported in detail previously and are summarized here.

Methods

Granulocyte agglutination. Granulocyte agglutination (GA) was done as previously described[5,17,18] using microtiter trays The results were read after five hours of incubation at 30 °C. The strength of reactivity was graded as 0, 1, 2, 3, or 4, according to the percentage of granulocytes that agglutinated.

Lymphocytotoxicity. Lymphocytotoxicity (LC) was done using the standard National Institutes of Health method.[19] The strength of the reaction in the crossmatch was graded as 0, 1, 2, 4, 6, or 8, based on the percentage of stained lymphocytes. Sera that had an incompatible LC crossmatch against donor cells were also screened against a panel of lymphocytes from 60 donors containing 20 HLA-A locus, 31 B locus, and eight C locus antigens. The percentage of the 60 panel cells that reacted was recorded, and the specificity of the HLA antibody reactivity determined by analyzing this pattern of reactivity.

Granulocytotoxicity. For granulocytotoxicity (GC), the method of Hasegawa et al[20] was used, except that blood for preparation of the cell suspension was collected in acid-citrate-dextrose (ACD), granulocytes were isolated using a double-density Ficoll-Hypaque gradient,[5] not papainized, and the sensitization phase of incubation was carried out at 22 °C and 37 °C. The test was considered positive when more than 40% of cells were stained. The strength of reactivity was graded as 0, 1, 2, 4, 6, or 8 in relation to the percentage of stained granulocytes.

Granulocyte immunofluorescence. Granulocyte immunofluorescence (GIF) was done essentially as described by Verheugt et al,[21] except that granulocytes were isolated using the double-density Ficoll-Hypaque gradient[5] and not subjected to hypotonic lysis of red cells.

Antibody-dependent lymphocyte-mediated granulocytotoxicity. This was our modification of the method of Logue et al.[22] Antibody-dependent lymphocyte-mediated granulocytotoxicity (ADLG) has been described in detail[23] and is summarized here. Blood was drawn into heparin, and the mononuclear cells were isolated by a double-density Ficoll-Hypaque gradient separation. Phagocytic cells were removed by carbonyl iron, and the lymphocytes were isolated, washed, and suspended in McCoy's medium containing 15% fetal bovine serum. Granulocytes were isolated from the double gradient, washed, labeled with 200 μCi of sodium ^{51}chromate, washed again, and resuspended in phosphate-buffered saline. The reaction mixture consisting of 10 μL of test or control serum and 10 μL of ^{51}Cr granulocyte suspension was incubated in U-bottom microtiter plates for 30 minutes at 37 °C in 5% CO_2. Then 130 μL of the lymphocyte suspension was added to give an effector-target cell ratio of 50:1. The mixture was then incubated for four hours at 37 °C, 50 μL of cell free supernatant was removed, radioactivity measured, and the percentage of cytotoxicity calculated. Controls included the following: (1) sodium dodecyl sulfate-treated granulocytes to measure the total release of ^{51}Cr; (2) AB serum from a nontransfused male to measure spontaneous release of ^{51}Cr in the presence and absence of lymphocytes; (3) O negative control serum; (4) autologous serum from the granulocyte donor; (5) positive control serum (anti-NA1, anti-NB1). Previous studies established a mean of 2.7% radioactivity released (cytotoxicity) in normal subjects.[23] A percentage of granulocytotoxicity greater than 2 SD above this mean was considered positive (cytotoxicity >10%) if the negative control values were within 2 SD of the normal mean.

Studies Using ^{111}Indium Granulocytes

Preparation and labeling of granulocytes. Granulocytes for ^{111}In labeling were processed, as previously described,[16] with the use of a discontinuous Ficoll-Hypaque gradient from 125 mL ACD whole blood obtained by phlebotomy The suspension contained an average of 1×10^8 granulocytes with less than 1% mononuclear leukocytes and less than 5% red blood cells.

Indium-111-oxine was prepared as previously published[16] The granulocyte suspension was incubated with indium-111-oxine at room temperature for 20 minutes with occasional gentle mixing and then infused into the recipient.[16] The small amount of labeled cell suspension remaining in the vial was used to determine labeling efficiency and the number of cells injected.

Determination of intravascular recovery and t½ Blood samples were obtained from the recipient at approximately ten minutes, 30 minutes, and 1, 2, 3, 4, 5, and 6 hours after injection, and the granulocyte-associated radioactivity was determined.[16] Calculation of the percentage of injected granulocytes recovered in the circulation and their intravascular half-life (t½) was based on total cell-associated activity injected and the recipient's blood volume as previously described.[16]

Body scans. Approximately 20 hours after injection of the indium-labeled granulocytes, the subjects underwent total body scanning using a Cleon body scanner (Union Carbide, Danbury, Conn). With this technique, indium-labeled granulocytes are normally seen in the liver and spleen. Thus the scans were classified as follows. true negative—radioactivity in liver and spleen and no evidence of clinical inflammation found on follow-up; true positive—radioactivity in liver and spleen and at the site of inflammation present at the time of the scan; false positive—radioactivity in liver, spleen, and other sites in the absence of inflammation; false negative—radioactivity in the liver and spleen, with none at a site of inflammation present at the time of the scan. The presence or absence of inflammation was determined by continued follow-up of the patients and review of their entire clinical course by one of us (J. McCullough, C. Ludvigsen, L. Forstrom), who did not know the results of the scan when making the final decision regarding inflammation.

Statistical analysis Patients were grouped based on the kind of antibody present and the presence or absence of neutropenia Each group of patients with antibodies was compared with patients matched for the presence or absence of neutropenia but with no antibodies. The mean value for recovery and t½ for each group was determined, and differences between groups were evaluated by two-sample Student's *t* test. The degree of the linear relationship between kinetics values (percentage of recovery and t½) and the strength of individual crossmatch scores was determined using a Pearson Correlation Coefficient test. Multiple regression analysis was used to examine the predictability of the association between combinations of crossmatch results and the kinetics values The differences in kinetics values among the groups of patients who received HLA-mismatched transfusions were tested by two-sample Student's *t* test. The relationship between positive and negative results in the five leukocyte antibody tests was determined using two-by-two contingency tables. The statistical significance was calculated using chi-square with the Yates correction factor. Differences in all tests were considered significant if $P < .05$.

RESULTS

A total of 93 indium studies (partial results of 58 were previously reported in reference 17) was done in 38 adult and 45 pediatric patients (some patients were studied more than once). The patients had a variety of diseases, including aplastic anemia, acute or chronic leukemia, solid tumors, postoperative suspected localized infection or sepsis, or were post bone marrow transplant. Some patients were receiving platelet or granulocyte transfusions, and some were undergoing an indium scan to detect suspected occult infection. Leukocyte antibody testing and body scans were done in all 93 studies, and granulocyte kinetics data were obtained in 53 studies.

Intravascular kinetics studies. In our previous experience[17] and in analyzing the present data, neutropenic patients had significantly reduced granulocyte recovery, and the presence of documented infection did not influence the intravascular recovery or t½. Therefore, patients in the present study were categorized based on the presence or absence of neutropenia (<1,000 PMN/μL), but not infection. In general, these patients were severely neutropenic. During 18 studies (Figs 1 and 2), the neutrophil count was known to be less than 1,000/μL but was not quantitated further. In the other 30 studies, the neutrophil counts were as follows: 1,000 to 500/μL, three studies; 500 to 200/μL, nine studies; and <200/μL, 18 studies.

In the 53 studies involving intravascular kinetics of indium granulocytes, no antibodies were detected in any of the five assays in 18 studies, nine in neutropenic, and nine in nonneutropenic patients (Figs 1 and 2). The average intravascular recovery and t½ in nonneutropenic patients (30.8% and 5.6 hours) were similar to our previous results in nonneutropenic patients without antibodies[17] and in normal subjects who received autologous granulocytes.[16] In neutropenic patients, the average intravascular recovery (10.7%) and t½ (5.4 hours) were also similar to our previous data on neutropenic patients.[17] In 18 studies, antibodies were detected by only one of the five methods, and in 17 studies, antibodies were detected by two or more methods (Figs 1 and 2). Compared with patients with no antibodies, there was a significant reduction in granulocyte recovery and t½ in the one nonneutropenic patient with only a GA antibody (6.7% *v* 30.8%; 0.3 hours *v* 5.6 hours) and in the four nonneutropenic patients who had a combination of GA plus other antibodies (9.7% *v* 30.8%; 0.3 hours *v* 5.6 hours). A statistically significant decrease in t½, but not recovery, also occurred in neutropenic patients with GA antibodies alone or in combination with others. Because of this demonstrated effect of GA antibodies, those patients were removed from the other groups in the remainder of this analysis.

One nonneutropenic patient had antibodies detected by GIF and other methods. The recovery (16.2%) and the t½ (1.4 hours) in this patient were significantly less than in comparable patients with no antibodies. The average recoveries in all other groups were not significantly less than in patients without antibodies. It should be emphasized that in the nine patients who had LC (HLA) antibodies alone or in combination with other antibodies, there was no significant reduction in the intravascular recovery or t½ of incompatible granulocytes when there was no accompanying granulocyte agglutinating activity (Figs 1 and 2). In four groups of neutropenic patients, either the recovery or t½ values were significantly higher than in the control patients with no

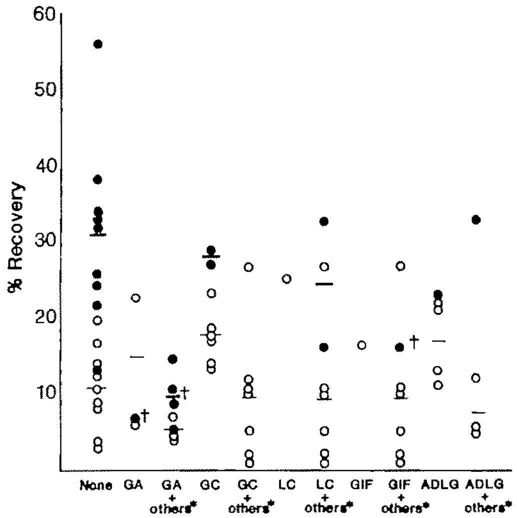

Fig 1. Intravascular recovery (%) of ¹¹¹indium granulocytes in 53 studies in patients with no or various combinations of leukocyte antibodies. *P* values represent comparison of the particular antibody group with patients with no antibodies. ●, PMN > 1000; —— = x̄; O, PMN < 1000; —— — x̄; *excludes GA antibodies; †, p < .01.

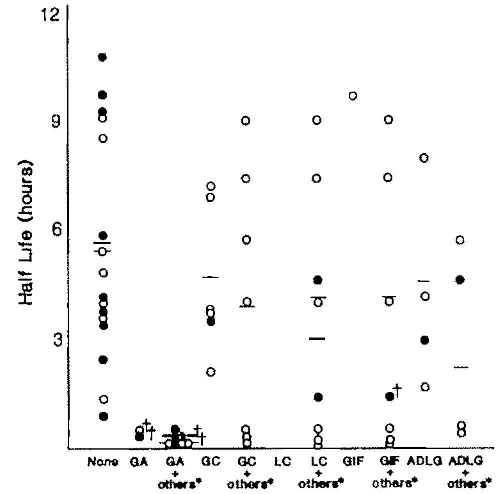

Fig 2. Survival (hours t½) of ¹¹¹indium granulocytes in 53 studies in patients with no or various combinations of leukocyte antibodies. *P* values represent comparison of the particular antibody group with patients with no antibodies. ●, PMN > 1000; —— = x̄; O, PMN < 1000; —— — x̄; *excludes GA antibodies; †, p < .01.

antibodies (Figs 1 and 2). In several of these situations, the data involved only one study. Six of the 14 patients involved had acute myelocytic leukemia and seven were infected. However, none had overwhelming infections or sepsis. No common features were apparent in these patients. Thus we believe that these statistically significant differences are probably due to the difficulty in standardizing all variables in these clinically ill patients and do not represent a causal relationship.

In order to analyze the relationship between multiple antibodies and the recovery or t½ of indium granulocytes, a multiple regression analysis was done comparing all possible combinations of antibodies with the recovery and t½. The presence of both GA and GIF antibodies had the best correlation with decreased recovery ($R^2 = .49$; $P < .001$); however, most of this effect was due to the GIF testing (GIF, $R^2 = .47$; GA, $R^2 = .28$). The combination of GA and GIF also had the best correlation with survival ($R^2 = .73$; $P < .001$); however, the GA and GIF made similar contributions (GA, $R^2 = .61$; GIF, $R^2 = .58$). In neutropenic patients, no combination of antibodies gave a correlation with recovery better than $R^2 = .26$.

The relationship between the strength of reactivity of the antibody and the recovery or t½ was analyzed. The Pearson Correlation Coefficient was used to test individual variables, that is, sera reactive in only one of the assays. Because the Pearson Correlation Coefficient requires a sample size of at least three, only GC testing could be analyzed. There was no significant relationship between the strength of GC reactivity and the percentage of recovery ($P = .34$) or the t½ ($P = .29$). Multiple regression analysis was used to study sera reactive in more than one assay. In nonneutropenic patients, there was a significant relationship between the strength of reactivity in the combination of GIF and LC assays and the recovery ($R^2 = .62$; $P = .001$). The strength of reactivity in the GIF alone was significantly related to the t½ ($R^2 = .60$; $P < .001$). Similar tests in neutropenic patients did not yield significant relationships possibly because the values for recovery in patients without antibodies were low and many studies would be necessary to show statistically significant differences.

Because of the relationship between the strength of reactivity of the LC and GIF with granulocyte recovery, and because of the wide availability of HLA (LC) testing, the role of HLA was investigated in more detail in several ways. The extent of reactivity of an HLA antibody can be determined not only by the degree of cytotoxicity against the individual donor cells in the crossmatch test, but also by the percentage of different random panel donors whose cells react. Those sera that reacted with 60% of different donors' lymphocytes were arbitrarily disignated as highly reactive. Of 13 sera that reacted with <60% of panel donors, few reacted by GA, GIF, GC, or ADLG (Table 1), and the granulocyte recovery and t½ were normal in three of four patients studied. Of 15 sera that reacted with >60% of panel donors, 14 (93%) also reacted by GIF. This was consistent with the observation that there was a statistically significant relationship between LC and GIF results ($P < .001$) but not between LC and other methods ($P > .05$). The granulocyte recovery and t½ were reduced in six of the eight patients studied whose sera were highly reactive by LC. The remaining two sera associated with the normal recovery and t½ did not contain GA antibodies. However, three of the six sera associated with reduced recovery and t½ were also GA negative.

Table 1. Relationship of Reactivity of Sera In the LC (HLA) Test to the Reactivity In Other Tests and to Reduced Granulocyte Recovery (R) and Survival (t½)

Panel Donors Reactive (%)	Sera Reactive by (%)					Reduced R and t½
	n	GA	GIF	GC	ADLG	
<60	13	8	23	38	31	1 of 4
>60	15	33	93	47	20	6 of 8

The effect of HLA antigen differences between donor and recipient was analyzed in combinations in which the donor's cells contained an antigen lacking in the recipient. The recovery and t½ for these combinations was compared with similar patients in whom either the HLA types of donor and patient were identical or the donor cells lacked an antigen that the recipient's cells contained.

There were 166 observations in which the patient's serum contained antibodies, but donor cells did not contain an HLA antigen lacking in the recipient (called matched, Table 2). There were 34 observations in which the patient's serum reacted against the donor's cells in at least one of the five assays and the donor cells contained an HLA antigen lacking in the recipient (called mismatched, Table 2). Significant findings involved HLA-A2 and BW44. There was a reduced t½ in the seven patients who received HLA-A2–incompatible granulocytes and reduced recovery and t½ in the three patients who received HLA-BW44–incompatible granulo-

Table 2. Influence of Donor–Recipient Mismatching for Selected HLA Antigens on the Recovery and Survival of 111-Indium Granulocytes

HLA Antigen	Recovery (%)				t½ (hours)			
	Matched Donor Recipient		Mismatched Donor Recipient		Matched Donor Recipient		Mismatched Donor Recipient	
	n	%	n	%	n	Hours	n	Hours
A1	16	12.5	4	14.2	13	2.1	4	2 4
A2	12	14.7	8	10.1	10	3 2	7	0.6*
A3	18	23.8	2	11.6	0	—	—	—
A24	15	12.9	5	12 6	13	2.2	4	2.1
B7	17	12.6	3	14 4	15	2.2	2	2 2
B8	18	13.9	2	3.8	15	2.4	2	0.4
BW44	17	14.7	3	2 4*	14	2 6	3	0.2*
B15	19	12.4	1	21.3	16	2.2	1	1.6
B35	17	12.0	3	17 7	15	2 3	2	.9
B40	17	11.8	3	19.1	15	1 9	2	3 8
Total	166		34		126		27	

Results shown are from 34 studies involving 18 patients whose serum reacted in one or more of the leukocyte antibody assays compared with 166 studies in 20 patients with antibodies in one or more leukocyte antibody assays and matched for selected HLA antigens.

*$P < .05$.

Table 3. Influence of Donor–Recipient Mismatching for HLA Antigens on Recovery and Survival of 111-Indium Granulocytes

		Sera Reactivity by							Patients With No Antibodies		Patient With HLA Antibody	
Patient	Neutropenic	GA	GC	LC	GIF	ADLG	Antibody	n	Recovery	t½	Recovery	t½
1	Yes	−	−	+	+	+	A2	9	10.7	5.4	5.1	0.5
2	Yes	−	+	+	+	−	A24	9	10.7	5.4	2.2	0.2
3	No	−	−	+	+	−	A2	9	30 8	5 6	16.2	1.4
4	Yes	−	+	+	+	−	A24	9	10.7	5.4	26.5	7.4

The serum of the four patients in the study contained a specific HLA antibody.

cytes. Both the recovery and t½ in HLA-B8 mismatched were reduced but not statistically significant ($P = .06$). Because of the observed effect of GA antibodies on recovery (Fig 1), the six patients with reactivity in the GA assay were excluded and the data on the remaining 12 studies was reanalyzed. Similar results were obtained; that is, there was a significant reduction in recovery and t½ with HLA-A2 and BW44 positive cells (data not shown).

Sera that react by LC often have broad reactivity and their HLA antigen specificity cannot be determined. Four patients who had HLA antibodies of clear specificity received granulocytes that contained the corresponding antigen; HLA-A2 (two cases) and A24 (two cases) (Table 3). None of the patients had GA reactivity, so that was not a confusing factor. In three patients (1 through 3), two with anti–HLA-A2 and one with anti–HLA-A24, it appears that the recovery of the incompatible donor cells was reduced and the t½ was shortened, although P values were not calculated because of the small number of studies done.

Tissue localization. Ninety-three indium scans were done, 45 of which were in patients determined to have localized inflammation at the time of the study. No antibody was detected by any of the five methods in 27 of the 93 studies. Thirty-two studies were done in patients who had an antibody detected by only one of the five methods (GA, - four; GC, 12; LC, - four; GIF, one; and ADLG, 11). (Table 4). Thirty-four other patients had antibodies detectable in more than one assay.

One of the patients with only a GA antibody had a true positive scan. She was a 4-year-old female with acute myelogenous leukemia, esophagitis, and colitis. There was localization of indium-granulocytes in the esophagus and colon. This is the only true positive scan we have seen in the presence of a GA antibody in more than four years of study. The antibody reacted weakly and gave an unusual, loose-appearing agglutination with only a few cells in each agglutinate, which may account for their ability to localize in vivo.

GA antibodies were present with others in 12 studies (Table 4). Six were true negatives and the previously mentioned child had a second positive scan at a time when the serum reacted by GC as well as by GA. Two patients had false negative scans. One had clinically apparent submandibular cellulitis and one, osteomyelitis at a leg amputation stump. A third patient is classified as both false negative and false positive. This 22-year-old female post renal transplant because of juvenile-onset diabetes had an intra-abdominal abscess at which indium granulocytes failed to localize (false negative) but showed pulmonary sequestration of indium granulocytes in the absence of evidence of pulmonary inflammation (false positive). Two additional patients with GA antibodies had abnormal pulmonary sequestration of indium granulocytes, and thus were classified as false positives. One was a 72-year-old man with chronic autoimmune neutropenia (Fig 3), whose serum reacted in the GA, GC, GIF, and ADLG tests. The intravascular recovery of granulocytes was reduced to 4.6% (normal, 10.7%), and their survival was short (t½, 0.2 hours; normal, 5.4 hours). Severe abnormal bilateral pulmonary uptake of indium granulocytes was apparent throughout the 24 hours postinjection. The other was a 9-year-old female post bone marrow transplantation for acute myelogenous leukemia. Neither patient had evidence of pulmonary inflammation and neither was on any drug that was likely to cause pulmonary toxicity. All of these patients who had false negative or false positive scans had serum reactivity in the GA and GIF assays. Many patients whose serum reacted in one or more other tests had true positive scans and none had false negative or false positive scans (Table 4).

DISCUSSION

In this study, granulocyte agglutinating antibodies alone and in combination with other leukocyte antibodies were

Table 4. Results of Indium Body Scans

Antibody	n	True Negative	True Positive	False Negative	False Positive
GA	4	3	1‡		
GA + others	12†	6	1‡	2½	2½
GC	12	6	6		
GC + others*	12†	7	5		
LC	4	2	2		
LC + others*	10†		2		
GIF	1	—	1		
GIF + others*	13†	11	2		
ADLG	11	5	6		
ADLG + others*	9†	7	2		

Results are shown from 32 patients with antibodies detected by only one of the five methods and 34 patients with antibodies detected in different combinations of two or more methods.

*Excludes GA antibodies.

†These studies represent 34 patients. Because the data is grouped to show different combinations of antibodies, the total appears greater.

‡These two studies were done on the same patient at different times.

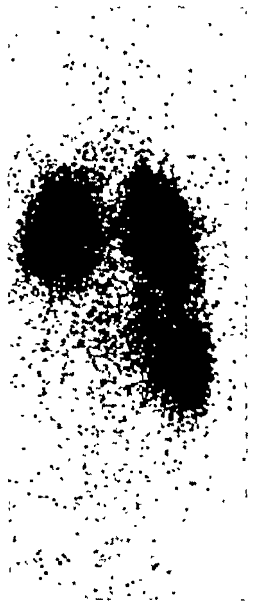

Fig 3. Indium-111 scan of a 72-year-old male with chronic neutropenia and GA, GC, GIF, and ADLG antibodies that reacted with the donor's cells. There is extensive abnormal bilateral localization of ^{111}In-labeled granulocytes in the lungs.

associated with decreased intravascular recovery and survival of incompatible granulocytes. In addition, antibodies detected by GA in combination with GIF were associated with failure of granulocytes to localize at sites of inflammation or with abnormal pulmonary sequestration, or both.

Analysis of the data in this manner did not indicate an important role for LC testing and the HLA system in granulocyte destruction, lack of migration to inflammatory sites, or abnormal pulmonary sequestration. However, because HLA antigens are found on granulocytes and there is widespread use of LC testing, we analyzed donor recipient combinations in which the donor's granulocytes contained HLA antigens lacking in the recipient. In patients whose serum reacted in one or more of the antibody assays, mismatching for HLA-A2, B8, or BW44 was associated with reduced recovery and survival of injected granulocytes. In four patients it was possible to examine the effect of the specific HLA antibodies anti-A2 and A24. There was a strong suggestion that these antibodies were associated with a reduced recovery and survival of donor granulocytes containing the corresponding antigen. Sera reactive by LC (HLA) against a broad range of panel donors (>60%) also were associated with reduced recovery and t½. There was a statistically significant association between reactivity of these sera and reactivity by GIF. Thus the combination of LC and GIF may define a group of highly immunized patients in whom the recovery and survival of injected granulocytes are reduced.

Ungerleider et al[14] found that neither the increment in granulocyte count after transfusion nor transfusion reactions were related to the presence of leukocyte antibodies detected by GC, LC, leukoagglutination, or capillary agglutination. The major shortcoming of their study, however, was the use of the posttransfusion granulocyte count to indicate a successful transfusion in granulocytopenic patients. Because of the effect of many patient variables on granulocyte counts in this range, this is probably not a satisfactory method of evaluating a single injection of granulocytes.

Two studies in dogs have established that prior exposure to blood from the granulocyte donor caused a decreased posttransfusion increment and migration into a skin chamber.[24,25] In one study,[24] testing by LC revealed antibodies in only 30% of the immunized dogs, while in the second study,[25] most immunized dogs had antibodies detected by LC, GC, and agglutination. Thus the optimum serologic tests to detect immunization in this dog model were not revealed from these studies. Subsequently, however, Chow and Epstein,[13] using a neutropenic dog model, showed that transfusions of granulocytes incompatible by GIF resulted in significantly lower increases in peripheral granulocyte count than in compatible transfusions. There was not a good correlation of granulocyte increment with lymphocytotoxicity or leukoagglutination.

Studies in humans also indicate that antibodies detected by immunofluorescence may have clinical significance. In 25 patients receiving granulocyte transfusions because of gram-negative sepsis, the presence of GIF antibodies was associated with death in five of six and their absence associated with recovery in 12 of 13.[12] This relationship was not found when clinical outcome was compared with lymphocytotoxicity (HLA) antibody results.

Dutcher et al[26] reported that indium-labeled granulocytes localized at sites of infection in 20/20 nonimmunized patients but in only 3/14 immunized patients. Alloimmunization also significantly increased pulmonary retention of indium-labeled granulocytes during the 30 minutes after injection.[27] In both studies, immunization was defined as the presence of HLA (LC) antibody. However, most patients had both lymphocytotoxic and leukoagglutinating antibodies so that the relative role of HLA and granulocyte antibodies could not be established.

Our studies and those of others summarized here do not establish which antigen system(s) is (are) involved in interference with the in vivo fate of granulocytes. GA detects neutrophil specific and some HLA antigens; GIF detects both neutrophil specific and HLA antigens, whereas LC primarily detects HLA antigens.

This study indicates that immune destruction of granulocytes can be predicted by the GA test, or even more accurately by the combination of GA and GIF tests. The association between LC and GIF reactivity and the observation of reduced recovery and t½ when some HLA antigens were mismatched suggests that LC antibodies and the HLA system are involved in granulocyte destruction. However, this is not well predicted by the usual interpretation of the LC assay. It appears that at present, the GA assay, if possible supplemented by the GIF, gives the best prediction of in vivo granulocyte kinetics. Possibly testing sera by LC against a large number of panel cells can also be used to identify highly immunized patients in whom granulocyte recovery and survival will be reduced.

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CONCISE REPORT

Autologous Transplantation of Blood-Derived Hemopoietic Stem Cells After Myeloablative Therapy in a Patient With Burkitt's Lymphoma

By Martin Körbling, Bernd Dörken, Anthony D. Ho, Antonio Pezzutto, Werner Hunstein, and Theodor M. Fliedner

A patient with Burkitt's lymphoma in complete remission received myeloablative consolidation treatment with superfractionated total body irradation (1,320 rad) and cyclophosphamide (200 mg/kg) followed by autologous transplantation of previously harvested and cryopreserved blood-derived hemopoietic stem cells. Seven successive leukaphereses were performed to yield a total of 55.2×10^9 mononuclear cells (MNC) comprising 15.1×10^6 CFU-GM or 4.34×10^6 CFU-GEMM. Following autologous blood stem cell transplantation (ABSCT), reconstitution of all cell lines occurred very rapidly, ie, 1,000 leucocytes per µL were reached after nine days, 500 granulocytes and 50,000 platelets per µL after ten days. B cells reached normal values around day 35 post-transplantation. CFU-GM first appeared in the circulating blood exhibiting an enormous overshoot. Some days later CFU-GM also appeared in the marrow. The kinetics and pattern of hemopoietic reconstitution after myeloablative treatment and ABSCT provide clear evidence that blood-derived hemopoietic stem cells are capable of completely restoring hemopoietic function in man. A possible reconstitutive advantage of blood over marrow-derived stem cells is discussed.

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EVIDENCE in rodents,[1] canines,[2] and nonhuman primates[3] indicates that hemopoietic progenitor cells capable of repopulating an aplastic marrow and maintaining lymphohemopoiesis circulate in the peripheral blood. Goodman and Hodgson first showed that lethally irradiated mice were fully reconstituted after transplantation of blood-derived leukocytes from closely related donor mice with identification of donor-type cells in the recipient.[1] This was also the case in the lymphopoietic system, as was further demonstrated in the canine model.[4]

At present, there is evidence that hemopoietic engraftment can be achieved with human peripheral blood leukocytes, although those clinical findings are controversial. From mice studies, some data suggest that circulating hemopoietic stem cells have a limited potential for self-renewal and therefore cannot maintain hemopoietic function as do marrow-derived stem cells.[5] If this applies to the human situation, the concept of replacing marrow by blood-derived stem cells would not be clinically applicable. To define the reconstitutive potential of circulating human stem cells, we describe in the following the successful lymphohemopoietic reconstitution of a patient with Burkitt's lymphoma after myeloablative treatment and autologous transplantation of blood-derived hemopoietic stem cells.

CASE REPORT

A 38-year-old male was admitted to the Heidelberg University Hospital with a fist-size tumor on the right side of the neck. From the biopsy, the immunohistological diagnosis of B-lymphoblastic non-Hodgkin's lymphoma (Burkitt's type) was made. The clinical staging (ultrasound, computerized axial tomography [CAT] scan, gallium-67 scans) subsequently performed revealed further abdominal tumor manifestation resulting in a stage III disease (St Jude classification). The induction treatment regimen consisted of two cycles of cyclophosphamide (1,000 mg/m^2 intravenously [IV]), vincristine (1.4 mg/m^2 IV), methotrexate (12.5 mg/m^2 IV), and prednisolone (1,000 mg/m^2 IV) together with two intrathecal administrations of methotrexate (12.5 mg/m^2) per cycle (COMP). Autologous marrow transplantation was then considered as intensive consolidation treatment. Since a regular multiple marrow aspiration from the pelvic site was not possible because of hyperostosis with a variable degree, we decided to use blood-derived, rather than marrow-derived, stem cells for engraftment. Approval for this experimental treatment was obtained from the hospital human investigational review board and appropriate informed written consent was given by the patient. Bone marrow biopsy and examination of the CSF did not show any sign of tumor cell involvement. There was no histological evidence for myelofibrosis and no clinical evidence for extramedullary hemopoiesis. The patient had received no prior radiation.

MATERIALS AND METHODS

Stem cell harvest. Sixteen days after completion of COMP treatment, blood stem cell collections were started. A total of seven consecutive leukaphereses were performed using a continuous-flow blood cell separator (FENWAL CS 3000, Fenwal Laboratories, Deerfield, Ill). The total blood volume processed per run was 10 liters at a flow rate of 70 mL/min. The centrifuge speed was adjusted to 1,000 rpm. Hydroxyethylstarch (HES) was used as RBC sedimentation enhancer at a total of 500 mL per run. The interval between stem cell harvests was between one and three days.[6]

Freezing of leukapheresis-derived stem cells. The 200 mL harvested cell suspension per run was concentrated and reduced in volume to 100 mL, and mixed with the same volume of Spinner-minimum essential medium (S-MEM) supplemented with 20% dimethyl sulfoxide (DMSO). The final 200 mL cell suspension was distributed over two polyolefine bags (DELMED, Canton, Mass) and frozen in a computerized freezer (CRYOSON BV-6, Cryoson Deutschland GmbH, Schöllkrippen, West Germany) to −100 °C. The frozen cells were stored in the liquid phase of nitrogen until use.

Pretransplant conditioning regimen. The pretransplant conditioning regimen consisted of a myeloablative dose of total body irradiation superfractionated over four days at 120 rad single doses up to a total of 1,320 rad (lungs, 900 rad). A linear accelerator was used as the radiation source. Three single doses were administered per day at 8 AM, noon, and 4 PM. Following irradiation, cyclophos-

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Table 1. Number of Mononuclear Cells, Granulocyte-Macrophage Committed Stem Cells, and Pluripotent Stem Cells Collected by Continuous-Flow Separation, Cryopreserved, and Eventually Transfused

	Pre-Freezing			Post-Freezing		
Run No.	MNC × 10^9	CFU-GM × 10^6	CFU-GEMM × 10^6	MNC × 10^9	CFU-GM × 10^6	CFU-GEMM × 10^6
1	6.8	2.0	0.6	↑	↑	↑
2	4.5	0.9	1.7			
3	6.4	ND	ND			
4	5.0	2.6	0.04			
5	15.0	3.9	1.0			
6	7.5	3.8	0.1			
7	10.0	1.9	0.9			
Total	55.2	15.1	4.34	51.9	18.8	9.3
Total per kg of body weight	0.77	0.21	0.06	0.72	0.26	0.13

ND, not determined because of technical problems.

phamide (50 mg/kg) was given on each of four consecutive days (total dose 200 mg/kg).

In vitro stem cell assay. The presence and concentration of hemopoietic progenitor cells was examined in each harvested cell suspension, in the peripheral blood, as well as in the marrow before (only blood stem cells) and after ABSCT. We used the human multilineage in vitro assay in methylcellulose (CFU-GEMM) recently described by Fauser and Messner[7] and modified according to Ash et al[8]

Immunologic studies The reconstitution of the B and T cell line after myeloablative treatment and ABSCT was followed by using specific T cell (OKT series, Ortho Diagnostic Systems, Raritan NJ) and B cell (HD 37) monoclonal antibodies. The latter antibody defines a B cell specific antigen that represents the broadest available marker for the B lineage (Cluster CD 19).

RESULTS

Cell harvest, freezing and autotransfusion. Prior to initial chemotherapy and ABSCT stem cell concentration in the peripheral blood was 26.7 CFU-GM per mL on the average (11.0 to 57.0 CFU-GM per mL; n = 3). CFU-GEMM were not detectable. Prior to each leukapheresis, the mean blood stem cell concentration was 375.3 CFU-GM per mL (154.6 to 406.0 CFU-GM per mL) and 146.4 CFU-GEMM per mL (4.7 to 601.6 CFU-GEMM per mL). As shown in Table 1, mononuclear cells including colony forming units (CFU) were collected by seven successive leukaphereses. The total number of MNC harvested so far was 55.2 × 10^9; that of CFU-GM, 15.1 × 10^6 or 4.34 × 10^6 CFU-GEMM. After a mean cryopreservation time of 74 days (63 to 93 days), the total number of MNC recovered was 51.9 × 10^9, that of CFU-GM 18.8 × 10^6 or 9.3 × 10^6 CFU-GEMM entailing an overall MNC recovery of 94% and a CFU recovery of > 100%. The number of MNC and of CFU-GM and CFU-GEMM transfused per kilogram of body weight is given in Table 1.

Reconstitution of leukocytes, platelets, and red blood precursor cells after ABSCT. Blood cell reconstitution after ABSCT occurred very rapidly and reached 1,000 leukocytes/μL on day 9, 500 polymorphonuclear cells/μL, and 50,000 platelets/μL on day 10 (Fig 1). Reticulocytes were first noted on day 11 (0.8 percent) increasing to 46 0/00 on day 18 (normal range, 0.5 to 1.5 percent of red blood cells). Platelets were substituted only twice. All three cell lines (reticulocytes not shown in Fig 1) exhibited a cellular overshoot in the early regeneration phase. At the first examination on day 8 after ABSCT, peripheral blood T cells were already within the normal range. However, the circulating B cell concentration showed a steady increase, eventually reaching normal values at day 34 (Fig 2).

Reconstitution of hemopoietic precursor cells after ABSCT. As shown in Fig 3, blood CFU-GM reappeared on day 5 preceded by an initial peak on day 1, presumably due to the transfusion of precursor cells with the stem cell graft. The curve peaked on day 14, exhibiting an enormous overshoot of about 70 times the pretreatment value. Marrow-derived CFU-GM reappeared later on day 7 after ABSCT without showing an overshoot phenomenon. CFU-GEMM showed up briefly in the peripheral blood, but reached a plateau in the marrow at a lower normal range from day 40 on.

DISCUSSION

Transplantation of circulating stem cells may have advantages over the use of marrow derived stem cells such as:

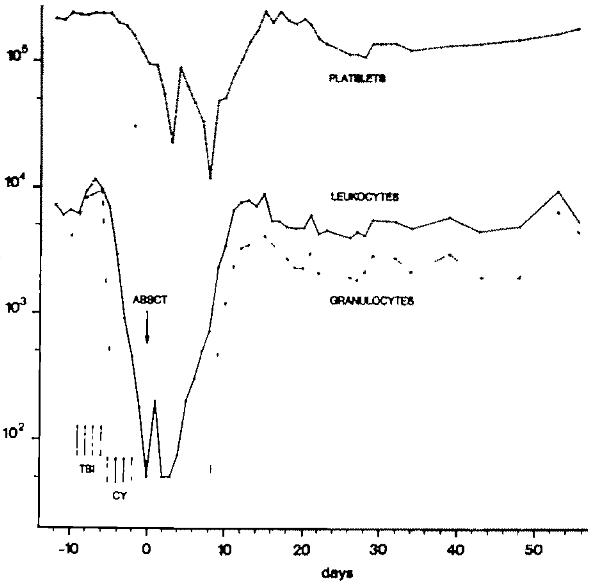

Fig 1. Kinetics of recovery of leukocytes, granulocytes, and platelets in the peripheral blood after ABSCT.

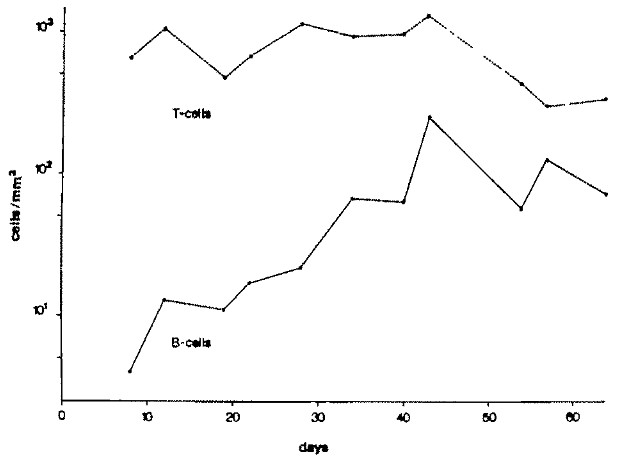

Fig 2. Kinetics of recovery of T and B cells in the peripheral blood after ABSCT (day of ABSCT = day 0).

(1) in patients at risk for general anaesthesia, continuous-flow apheresis offers an alternative and safe way for stem cell harvest;

(2) stem cell harvest is feasible in case of damage to the marrow collection site by previous radiotherapy or tumor involvement;

(3) hemopoietic reconstitution after myeloablative treatment seems to be more rapid for the WBC line, and therefore the aplasia-related risks in the early posttransplant period are lowered;

(4) the ratio between normal hemopoietic stem cells and clonogenic tumor cells in the peripheral blood of patients with malignant lymphohemopoietic disorders in remission might be in favor of the former, a hypothesis yet to be proven.

From the clinical point of view, blood stem cell transplantation to restore hemopoietic function after myeloablative treatment has to fulfill two major requirements: (1) hemopoietic reconstitution (including the lymphopoietic system) must be complete; and (2) the reconstituted hemopoietic function must be permanent.

In an extensive clinical study, Goldman et al were the first investigators to have clearly shown that blood-derived hemopoietic stem cells harvested in the chronic phase of patients with chronic myelogenous leukemia (CML) can re-establish hemopoietic function after myeloablative treatment for blast crisis.[10] Unfortunately, most stem cells so far collected and eventually transfused originate from a Ph^1-positive tumor cell clone, ie, their repopulating capability does not necessarily mean that "normal" stem cells act the same way. We attempted to make a step forward to "normality" by using stem cells collected in a transient chemotherapeutically induced Ph^1-negative phase.[11] Those normal Ph^1-negative blood-derived stem cells completely restored hemopoietic function without reappearance of the Ph^1 chromosome Unfortunately, the observation time after ABSCT was too short to be able to draw definite conclusions about the character and quality of the transfused stem cell clones. One attempt has been made to use syngeneic blood-derived stem cells for marrow repopulation. In this case, hemopoietic function was incomplete and has therefore failed so far, because of insufficient CFU-GM numbers transfused.[12] On the other hand, fatal graft *v* host disease following blood transfusions from normal donors into immunosuppressed patients has been reported, indicating allogeneic engraftment of accidentally transfused hemopoietic precursor cells.[13] Furthermore, the rate of successful marrow engraftment in patients with aplastic anemia is probably increased by the additional transfusion of "buffy coat" cells in terms of stem cell support.[14]

The present case provides clear evidence that complete

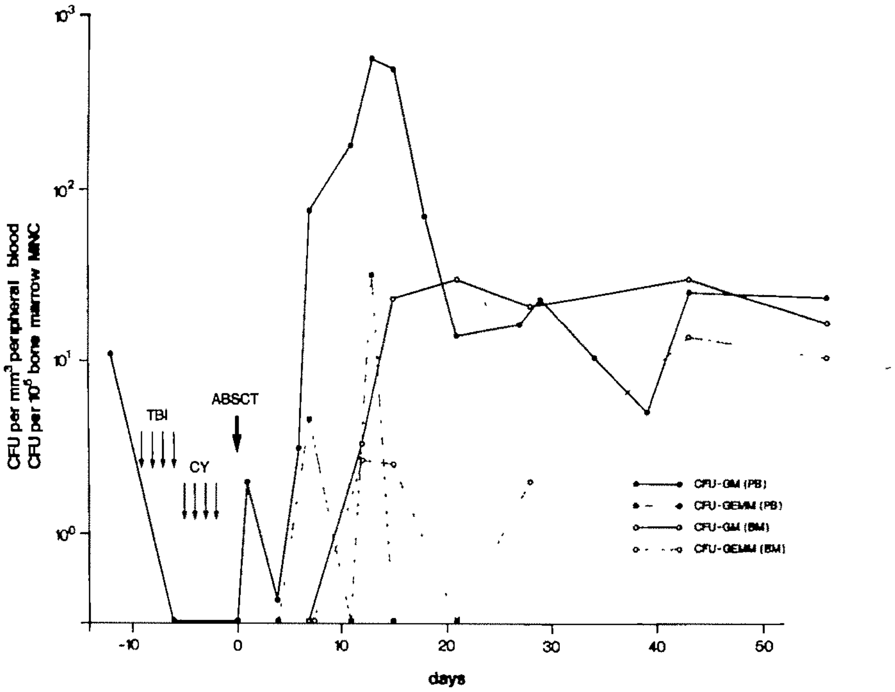

Fig 3. Kinetics of recovery of CFU-GM and CFU-GEMM in peripheral blood and bone marrow after ABSCT.

hemopoietic reconstitution can be achieved after myeloablative treatment using normal blood-derived stem cells including the lymphopoietic system. The minimal number of CFU-GM per kilogram body weight (based on canine transplantation data) needed for safe hemopoietic reconstitution was exceeded by a factor of two to five.[15,16] The high yield of leukapheresis-derived hemopoietic precursor cells reflects an expansion of the blood stem cell pool at the time of stem cell harvest. Since leukapheresis started just 2 weeks after completion of chemotherapy, stem cell harvest was probably performed during a chemotherapy-induced blood stem cell overshoot as described first by Richman et al.[17] On the other hand, blood stem cell concentration before initial chemotherapy and ABSCT was found to be at relatively normal level.

It it noteworthy that hemopoietic reconstitution occurred very rapidly, due to the high numbers of hemopoietic precursor cells transfused. The question whether blood-derived human stem cells exhibit a restorative advantage over marrow-derived ones is still speculative. From animal studies comes some evidence for a difference in the proportion of CFU-GM to pluripotent stem cells in cell suspensions obtained from blood and bone marrow. In canine studies, Appelbaum[18] calculated that for a successful engraftment, 2.4 times as many CFU-GM were required from autologous bone marrow than from peripheral blood, indicating a relatively higher pluripotent stem cell number per CFU-GM in blood than in bone marrow.[18] Evidence for a higher restorative potential of blood- *v* bone-marrow–derived precursor cells was described in the canine model for lymphopoiesis[18] as well as for lymphopoiesis and granulocytopoiesis.[19]

The potential of circulating stem cells to sustain hemopoiesis after myeloablative treatment and ABSCT is well documented in dogs. Blood stem cell transfused dogs were observed for as long as 3 years, after which they were killed and found to have a well-functioning hemopoiesis.[15] Our patient's blood and marrow cellularity is in the normal range for more than 7 months after ABSCT. Nevertheless, a long-term follow-up is needed to judge the quality of the blood-derived graft.

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CONCISE REPORT

Clonal Rearrangement and Expression of the T Cell Receptor β Gene and Involvement of the Breakpoint Cluster Region in Blast Crisis of CGL

By L.C. Chan, A.J. Furley, A.M. Ford, D.A. Yardumian, and M.F. Greaves

A case of lymphoid blast crisis of Ph^1-positive CGL is described in which the blast cells had an immature T cell phenotype, clonal rearrangement and expression of the T cell receptor β gene, and a rearrangement of the breakpoint cluster region (*bcr*) on chromosome 22. This case therefore provides definite evidence for transformation involving a common myeloid–T lineage progenitor, penetrance of the Ph^1 molecular alteration into the T cell lineage, and clonal selection in blast crisis at the level of a committed T lineage precursor.

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CHRONIC granulocytic leukemia (CGL) originates in a multipotential, hematopoietic stem cell.[1-3] Progeny of this transformed cell include granulocytic, erythroid, megakaryocytic, and monocytic cells, eosinophils, and B lymphocytes.[1,4] An involvement of the T cell lineage in Philadelphia (Ph^1)-positive CGL has, however, been difficult to establish; attempts to demonstrate the Ph^1 chromosome or monoclonality with respect to glucose 6PD in T cells from patients in chronic phase have yielded variable or equivocal results.[3,5] Immunophenotyping studies of CGL in blast crisis have shown a preponderance of myeloid and/or B cell precursor phenotypes[3,6-8] with only a few isolated cases of possible T cell lineage involvement.[9-12] Similarly, de novo ALL with Ph^1 chromosome have common ALL or B cell precursor phenotypes,[13] whereas Ph^1-positive T-ALL, though recorded,[11,14,15] is very rare. Two recent cases,[16,17] one of childhood T lymphoblastic lymphoma and the other of T-ALL, have provided evidence that the Ph^1 chromosome may arise as a secondary event in the evolution of these disorders.

Selective growth advantage of single B cell precursors in blast crisis of CGL has been demonstrated by clonal rearrangements of immunoglobulin genes.[18,19] The recent availability of molecular probes for the T cell receptor (TCR) β gene now provides a similar opportunity to explore T cell selection in CGL since this gene rearranges in a similar fashion to immunoglobulin[20] and is shown to be clonally rearranged and transcribed in T cell malignancies.[21] In addition, the cloning of a DNA probe that specifically recognizes rearrangements in the breakpoint cluster region (*bcr*) on chromosome 22 in CGL[22] provides a new and unique molecular marker to analyze particular cell types including T cells for their involvement in CGL. We recently identified a rare case of blast crisis of Ph^1 CGL in which the blast cells had the composite phenotype of thymic leukemia cells (cf T-ALL). We report here the detailed immunophenotype of this case and results for probing for rearrangement and expression of TCR β gene and also for involvement of *bcr*.

MATERIALS AND METHODS

Restriction enzymes *Bam*HI, *Eco*RI, *Hind*III, and *Bgl*II were supplied from Anglian Biotechnology Laboratories and used according to the manufacturer's specifications; $[\alpha\text{-}^{32}\mathrm{P}]$ dCTP (specific activity 300 Ci/mmol) was purchased from Amersham International. Two probes were used for the study: (1) a 300-bp *Bgl*II/*Stu*I constant region cut from Jurkat cDNA of TCR β gene[23] was kindly provided by Dr Tak Mak (Ontario Cancer Institute), and (2) a 1.2-kilobase *Bgl*II/*Hind*III *bcr* cloned fragment was kindly supplied by J. Groffen (Oncogene Scientific, Inc, New York).

A leukemic sample from patient J.H. was separated on a Ficoll-Hypaque gradient and immunophenotyped with a panel of antibodies.[24] DNA and RNA isolation, blotting, nick translation, hybridization, washing, and autoradiography were performed according to established procedures.[22,25] Placental DNA was used as a control for gene rearrangement studies, while RNA from a thymus taken from a 5-year-old girl undergoing cardiac surgery served as a control for RNA analysis.

RESULTS

The clinical and laboratory data of patient J.H. are shown in Table 1. The composite phenotypes of the leukemic blasts WT1$^+$(CD-7)$^+$, T11a(CD-2)$^+$, TdT$^+$, MY9$^-$, B4$^-$, and SmIg$^-$ indicates this case to be of early T cell precursor origin.

Hybridization of J.H.'s DNA with the TCR β gene probe shows clonal rearrangement of a TCR β gene allele of 25.0 kb coexisting with the remaining 22.7 kb germ line allele in the *Bam*HI digest (Fig 1A). The autoradiograph pattern of the *Eco*RI digest (Fig 1B) confirms that the rearranged allele derives from $C\beta_1$; this is shown by the creation of a new band of 11.4 kb coexisting with the remaining 10.5-kb germ line $C\beta_1$ allele. The smaller band of 4.5 kb in the *Eco*RI track represents $C\beta_2$ alleles, rearrangement of which would not be detectable as there exist *Eco*RI restriction sites both $5'$ and $3'$ of the $C\beta_2$ locus. The *Hind*III digest (Fig 1C) confirms noninvolvement of the $C\beta_2$ locus as it shows a single germ line of 7.5 kb; similarly, the smaller band of 3.4 kb represents $C\beta_1$ alleles, rearrangement of which would not be apparent as *Hind*III cuts $5'$ and $3'$ of the C locus. The status of the immunoglobulin heavy-chain locus was similarly examined using a human μ constant region probe ($C\mu$). No rearrangement was observed when a *Bam*HI digest of the patient's DNA was probed with the $C\mu$ probe (data not shown). The results from these analyses therefore reinforce the lineage affiliation of the leukemic blasts to be of T cell origin. Finally, the T cell lineage of the blast crisis was further

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Table 1. Clinical and Laboratory Data of CGL–T Cell Blast Crisis

Patient:	J.H.	
Age:	66	
Sex:	Male	
WBC count:	109 × 10^9/L	
% blasts:	PB, 11.0	
	BM, 71.0	
Hematologic diagnosis:	CGL blast crisis (patient presented 3 years previously with classical Ph^1 CML)	
Cytogenetics:	46, XY, t(9:22)	
Surface markers:	Bone marrow	
(1) T cell:	WT1(CD-7)*	66%
	T11a(CD-2)*	74%
	OKT4(CD-4)*	0%
	C3(CD-8)*	0%
	Na134(CD-1)*	0%
	SmIg	0%
(2) B cell:	B4(CD-19)*	0%
(3) Myeloid:	MY9	0%
(4) anti-CALLA:	AL2(CD-10)*	75%
(5) anti-HLA-DR:	DA2	21%
(6) Terminal transferase:	(TdT)	75%
Double staining:	WT1/TdT	78%
Immunophenotype:	Immature thymic	

*CD, cluster of differentiation with arbitrary number assigned (see *Immunology Today* 5:158–159, 1984).

supported by the evidence of TCR β gene transcripts (Fig 2) from an RNA blot; however, the size of the transcripts lies between the 1.3-kb-size message representing complete VDJC sequences and the 1.1-kb-size transcripts from DJC rearrangement as described in various T cell lines and fresh T-ALL leukemic samples.[26,27]

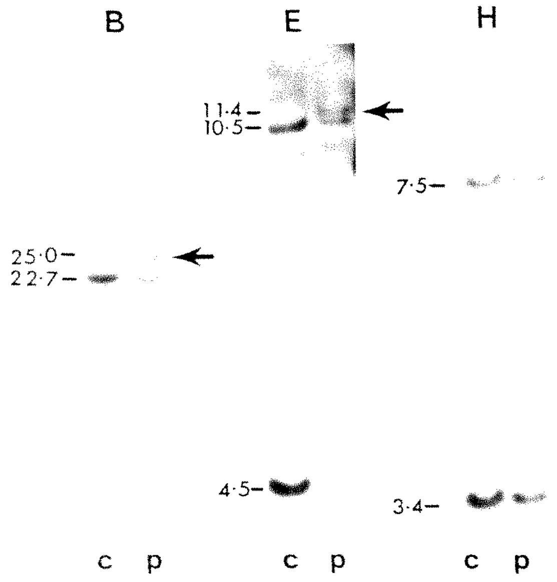

Fig 1. Autoradiographs of DNA digests hybridized with a TCR β constant region probe. B, *Bam*HI; E, *Eco*RI; H, *Hind*III. c, control (placental) DNA; p, patient's DNA. Sizes of DNA fragments are given in kilobases (kb). Clonally rearranged bands arrowed.

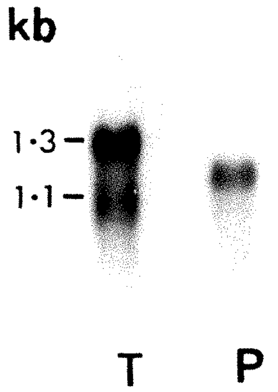

Fig 2. Autoradiography of RNA transcripts hybridized with a TCR β constant region probe. T, thymocyte RNA; P, patient RNA. Sizes of RNA transcripts are given in kilobases (kb).

To find out whether the same breakpoint cluster region in CMLs was involved in this case of T cell blast crisis, DNA of J.H. was analyzed by hybridization to a *bcr* probe. The involvement of the bcr locus on chromosome 22q is shown by the existence of a new rearranged *Bgl*II band of 3.2 kb together with a germ line *Bgl*II band of 5.0 kb representing the normal chromosome 22 (Fig 3); similarly, there is a new *Eco*RI band of 26.0 kb together with the normal 18.5-kb *bcr* allele. No rearrangement of *bcr* locus was seen in the *Bam*HI and *Hind*III digests, suggesting that the breakpoint in *bcr* on chromosome 22 lies upstream of the *Bam*HI site, as shown in Fig 4. The fact that only a single new band was detected in the *Eco*RI and *Bgl*II digests emphasizes that clonal rearrangement of *bcr* locus has occurred.

DISCUSSION

The involvement of T cells in CGL blast crisis phase of Ph^1-positive CGL is confirmed in the study. Although the composite immunophenotype of the leukemic cells in this case indicates that they were of thymic type, as observed in T-ALL, the demonstration of rearrangements in the TCR β

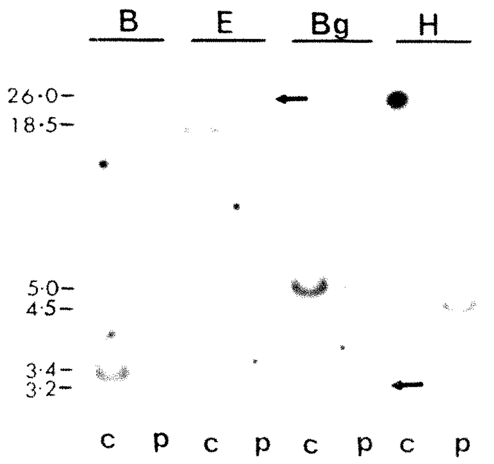

Fig 3. Autoradiograph of DNA digests hybridized with a *bcr* probe. B, *Bam*HI; E, *Eco*RI; Bg, *Bgl*II; H, *Hind*III. c, control DNA; p, patient's DNA. Sizes of DNA fragments are given in kilobases (kb). Clonally rearranged bands arrowed.

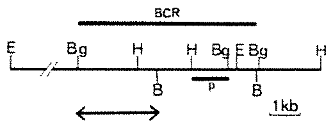

Fig 4. Restriction map of breakpoint cluster region (22). B, *Bam*HI, E, *Eco*RI; H, *Hind*III; Bg, *Bgl*II. BCR, Ph^1 translocation breakpoint cluster; p, 1.2-kb probe; ↔ denotes proposed region of break in patient J.H.

gene and the expression of TCR β gene transcripts reinforces this lineage affiliation. The intermediate-sized TCR β gene transcripts between 1.3 kb and 1.1 kb lead us to speculate that they may arise from an incomplete rearrangement. Furthermore, since the TCR β gene rearrangements were clearly monoclonal, we conclude that blast crisis in this patient involved clonal selection at the level of T cell precursors that had already rearranged the TCR β gene. This, then, parallels blast crisis with a common ALL phenotype in which lymphoblasts have clonal rearrangements of the immunoglobulin gene.[18,19] In addition, we found that *bcr* on chromosome 22 was rearranged, thus proving that the T lymphoblasts in this case were members of the Ph^1 leukemic clone.

We take these data as providing definite evidence for the involvement, albeit rare, of the T lineage in Ph^1-positive CML and presume that, in these cases, at least, the initial target cells for transformation descended from a common progenitor of the T and myeloid lineages. Additional proof of the existence of such a common progenitor comes from a recent report by Fauser et al[27] that demonstrates the presence of the Ph^1 chromosome in T lymphocytes within multilineage hematopoietic colonies (CFU-GEMM T) generated from bone marrow cells from a patient with Ph^1 CML.

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CONCISE REPORT

Ultraviolet Irradiation of Blood Prevents Transfusion-Induced Sensitization and Marrow Graft Rejection in Dogs

By H. Joachim Deeg, Joseph Aprile, Theodore C. Graham, Frederick R. Appelbaum, and Rainer Storb

In a canine model using DLA-identical littermate pairs, we have shown that a regimen of three transfusions of donor blood given 24, 17, and 10 days before transplant uniformly leads to marrow graft rejection, presumably due to sensitization to minor (non-DLA) histocompatibility antigens. Untransfused dogs uniformly achieve sustained engraftment. In the present study, we investigated whether the exposure of blood to ultraviolet (UV) light (220–300 nm) prior to transfusion prevented sensitization of the recipient and allowed for successful marrow engraftment. Ten dogs were each given three pretransplant transfusions from the marrow donor. Each transfusion consisted of 50 mL of whole blood exposed in vitro to UV light for a total of 1.35 J/cm^2. All ten dogs achieved engraftment. In contrast, all four dogs that had received sham-exposed transfusions rejected their grafts. In vitro studies revealed that although cell viability was not affected, leukocytes contained in UV-exposed blood were unable to function as stimulator cells in mixed leukocyte cultures or as accessory cells in mitogen-stimulated cultures. These data are consistent with the hypothesis that accessory cells are involved in transfusion-induced sensitization. We conclude that in vitro exposure of blood to UV light before transfusion prevents sensitization and allows for subsequent marrow engraftment.

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PATIENTS RECEIVING a marrow transplant for the treatment of severe aplastic anemia only rarely reject the graft if they are untransfused at the time of transplantation.[1] However, patients who have received transfusions before transplantation are at a high risk for graft rejection, even if the marrow donor is an HLA-identical sibling (see review[2]).

The adverse effect of pretransplant transfusions on the outcome of marrow transplantation had been predicted from animal studies (see reviews[3,4]). Untransfused dogs given 9.2 Gy of total body irradiation (TBI) and marrow grafts from siblings identical at the major histocompatibility complex (DLA) generally achieve sustained engraftment, whereas dogs transfused with whole blood from the marrow donor before grafting uniformly reject the graft as a result of transfusion-induced sensitization of the recipient against minor (non-DLA) antigens of the donor.[3] If, however, leukocyte-depleted red blood cells (RBC) or platelets are transfused, graft rejection is significantly reduced,[3] suggesting that leukocytes are responsible for sensitization. We hypothesized that the effect of transfused leukocytes on the recipient in vivo was comparable to the effect of stimulator leukocytes on responding cells in mixed leukocyte culture (MLC) in vitro. Since it has been shown that exposure of leukocytes to ultraviolet (UV) light abrogates their ability to function as stimulator cells in MLC,[5] we investigated whether in vitro exposure of blood to UV light abrogated its ability to sensitize in vivo.

MATERIALS AND METHODS

Dogs. Purebred and mongrel dogs, 6 to 12 months old, were purchased from commercial kennels and cared for as described.[3] Donor-recipient pairs were littermates, DLA-identical as determined by serologic typing for DLA-A, B, and C and mutual nonreactivity in MLC.[3]

Conditioning and marrow transplantation. Recipient dogs were prepared for transplantation by 9.2 Gy of TBI, and donor marrow cells, 2.8 ± 2.1 × 10^8/kg, were infused within 4 hours of TBI as described.[3] On days 1 and 2, viable leukocytes (18.2 ± 7 × 10^8/kg), obtained from the donor via arteriovenous shunt, were infused. No postgrafting immunosuppression was given. The postgrafting care has been described.[3] Engraftment was documented by rising granulocyte counts following the postirradiation nadir and by the presence of donor sex karyotype in metaphase spreads from bone marrow and peripheral blood or the conversion to the donor-type RBC antigen pattern.[3] Complete autopsies were carried out in all dogs that died.

Transfusion regimen. Transfusions of 50 mL of blood from the intended marrow donor were given 24, 17, and 10 days before transplantation. Blood was drawn via venipuncture into plastic syringes containing preservative-free heparin, 10 U/mL.

UV irradiation of blood. Whole blood was diluted 1:1.5 with Waymouth's minimal medium. Aliquots of 7.5 mL were placed in plastic dishes (Falcon #3003), for a layer of 1.5 mm thickness. Uncovered dishes, on a rotating platform, were exposed for 30 minutes to UV light (220–300 nm) from a germicidal lamp (General Electric) at 750 μW/cm^2 for 1.35 J/cm^2 as determined with a Black Ray shortwave UV meter (U.V. Products).[6] Blood was then recovered from the dishes and injected into the recipient dog. Sham-irradiated blood was exposed to visible light instead of UV light.

In vitro studies. Aliquots of UV- or sham-exposed blood were fractionated on Ficoll-Hypaque gradients, and interface cells (PBMC) were processed and used in MLC and mitogen cultures as described.[7] For further analysis, 100–300 × 10^6 PBMC were fractionated on discontinuous Percoll gradients. The two lowest density fractions (49.6% and 51.1% Percoll) were pooled, washed twice, suspended in Waymouth's medium and 10% fetal calf serum, and incubated on plastic dishes (Falcon #3003) at 37°C. After 18 hours, nonadherent cells, enriched for dendritic cells,[7] were resuspended at 10^6/mL in Waymouth's medium containing 20% normal canine serum. Purified lymphocytes were obtained from Percoll fraction 5 (F5).

RESULTS

Results are summarized in Table 1. All ten dogs given UV-exposed blood before transplantation had marrow

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Table 1. Results in Dogs Given 9.2 Gy TBI and Hematopoietic Grafts From DLA-Identical Littermates With or Without Three Preceding Transfusions of Whole Blood From the Marrow Donor

	Number of Dogs			
Type of Transfusion	Studied	With Graft Rejection	With Engraftment	% Engraftment
Whole blood, UV-irradiated	10	0	10*	100
Whole blood, sham-irradiated	4	4†	0	0
None	60‡	1	59	98
Whole blood, unmanipulated	21‡	21	0	0

*Three dogs died with septicemia at 8, 9, and 13 days, respectively. Engraftment was documented by rising granulocyte counts following the postirradiation nadir and by the presence of erythroid and myeloid precursor cells (total cellularity 10–25% of normal) on marrow samples obtained at autopsy. Seven dogs are surviving > 100 days; in these, sustained engraftment was documented by the presence of the donor sex karyotype in all metaphase spreads from bone marrow and peripheral blood (4 dogs) or the conversion to the donor erythrocyte antigen pattern (3 dogs).

†These dogs died on days 10, 11, 11, and 13, respectively, with septicemia. Marrow cellularity at autopsy was less than 5% in all dogs. Cells were composed of plasma cells and reticulum cells; hematopoietic precursor cells were absent.

‡The majority of these dogs were included in a previous report.[3]

engraftment. All four dogs transfused with sham-exposed blood rejected their grafts and died with marrow aplasia. Included for comparison are results from previous studies,[3,4] showing that untransfused dogs achieve engraftment, whereas dogs transfused with normal blood uniformly reject their grafts.

In vitro results are summarized in Fig 1. While ^{137}Cs-irradiated leukocytes were potent stimulators in MLC, UV-exposed leukocytes were not. As expected, no stimulation was seen with ^{137}Cs- or UV-exposed DLA-identical leukocytes. However, DLA-identical cells enriched for dendritic cells, in a quasi-autologous MLC, functioned as potent stimulators after ^{137}Cs but not after UV exposure. In addition, mitogen responsiveness of accessory cell–depleted lymphocytes (F5) was restored by the addition of normal or sham-exposed PBMC but not by UV-exposed PBMC.

DISCUSSION

We have shown in this study that dogs transfused with UV-exposed blood from the intended marrow donor uniformly achieved sustained engraftment of a subsequent marrow graft and thus behaved like untransfused dogs.[3] In contrast, dogs transfused with sham-exposed blood, as with unmanipulated blood,[3,4] uniformly rejected their grafts. Abrogation of the sensitizing ability of blood was not due to a destruction of cells but, presumably, to a modification of their immunogenicity. In vivo RBC survival (data not shown) was comparable to that of unmanipulated RBC, and in vitro studies revealed viable (viability >90%), structurally intact leukocytes.

We have shown previously that thoracic duct lymphocytes, devoid of accessory cells, and cotton-wool nonadherent cells depleted of Ia-positive accessory cells fail to induce sensitization.[4] We have also shown that purified monocytes are poor accessory cells, whereas dendritic cells provide potent stimulator and accessory function, which, similar to findings in other species,[8] is abrogated by UV exposure.[6,7] Lau et al[9] exploited this finding in a rat model of pancreatic islet cell transplantation and showed that pretransplant exposure of islets to UV light resulted in significant prolongation of graft survival. The implication was that UV exposure inactivated dendritic cells, thus eliminating a critical dendritic cell–dependent signal (from the graft) that normally triggers graft rejection by the recipient.

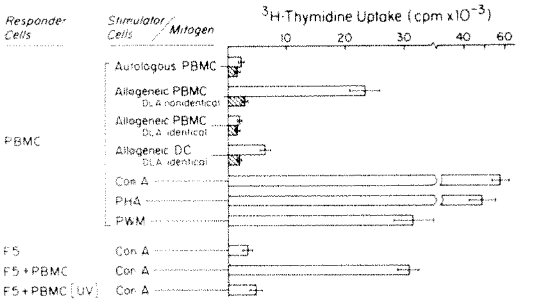

Fig 1. Proliferative responses of normal PBMC and purified lymphocytes (F5, obtained by Percoll gradient separation). Responder cells were stimulated by ^{137}Cs-irradiated (2,000 rad; open bars, control) or UV-exposed (1.35 J/cm^2; shaded bars) allogeneic stimulator cells (PBMC or PBMC enriched for dendritic cells [DC]), either from a DLA-identical sibling or from a DLA-nonidentical unrelated donor. In addition, responder cells were stimulated by the lectins concanavalin A (Con A), phytohemagglutinin (PHA), or pokeweed mitogen (PWM) at optimal concentrations. Where indicated, accessory cell–depleted purified lymphocytes (F5) were cocultured with 1 × 10^4 normal or UV-exposed PBMC. Viability of ^{137}Cs-irradiated cells, as determined by trypan blue uptake, was < 10%, compared to > 90% among cells exposed to UV light. The bars show the means and the —— lines the standard errors of the mean of 12 experiments for MLCs and 4 experiments for lectin-stimulated cultures.

The mechanism by which UV exposure modifies immunogenicity is not clear. Results in murine models suggest that the injection of UV-exposed cells leads to the activation of suppressor cells.[10] In the present study, however, we found no evidence for a role of suppressor cells (data not shown). Others have shown that UV exposure interferes with interleukin production,[13] but it is not clear why interleukins should not be provided in vivo by the transfusion recipient. Also, we have no evidence for a decrease of the expression of Ia-like antigen after UV exposure, as suggested by others,[11] although it is possible that Ia function was altered.[12] This, in turn, could result in defective presentation of minor (non-DLA) histocompatibility antigens to the recipient and a lack of sensitization. Alternatively, non-DLA antigens on donor cells could be modified by UV exposure, thus escaping recognition by the recipient. Therefore, we cannot implicate a single cell population in sensitization.

Regardless of the mechanism involved, the present results suggest that UV-exposed blood can be transfused without jeopardizing the success of a subsequent DLA-identical marrow graft. These observations may be relevant for transfusion support in general. Preliminary results in a canine platelet-transfusion model indicate that UV exposure of platelets can prevent refractoriness and facilitate platelet transfusion support.[14]

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CONCISE REPORT

Paroxysmal Nocturnal Hemoglobinuria Erythrocytes Are of Two Distinct Types: Positive or Negative for Acetylcholinesterase

By Michael E. Dockter and Martin Morrison

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired disorder. Erythrocytes isolated from PNH patients show increased sensitivity to complement and decreased acetylcholinesterase (AChE) activity. In this study, indirect immunofluorescence analysis of a monoclonal antibody specific for a surface epitope of human erythrocyte AChE is used to quantitate the content of this enzyme at the single-cell level. Flow-cytofluorimetric analysis of erythrocytes from normal donors indicates that all erythrocytes contain detectable levels of the surface epitope with a strong correlation between cell size and enzyme content. In contrast, erythrocytes from PNH patients show two distinct populations of erythrocytes; namely, those containing a normal content of AChE and a second population containing no detectable AChE. The AChE-negative population of cells is quantitatively complement-sensitive. These data support suggestions that PNH is a clonal disorder resulting in two distinct types of circulating erythrocytes. The abnormal clone produces cells that are both surface-AChE-negative and complement-sensitive. In addition, the method described provides an attractive alternative for the diagnosis and quantitative evaluation of abnormal erythrocytes in PNH patients.

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PAROXYSMAL nocturnal hemoglobinuria is an acquired hemolytic anemia most consistently characterized by the increased sensitivity of erythocytes, platelets, and granulocytes to complement-mediated lysis.[1] This results in intravascular hemolysis resulting in hemoglobinuria. Additional clinical manifestations of this disease may also include iron deficiency, prolonged bleeding, thromboses, abnormalities in renal functions, and neurologic abnormalities. The sensitivity of PNH erythrocytes to complement has been the basis for the diagnosis of this disease.[2,3] A second consistent feature of the red cells from PNH patients is the decreased specific activity of acetylcholinesterase,[4-8] while the Km for this enzyme remains unchanged.[9,10] A closer examination of PNH erythrocytes has indicated that the cells that are not sensitive to complement-mediated lysis have higher specific acetylcholinesterase activity than that of the whole cell population before lysis.[7,8] The simplest explanation of these results is that there are two populations of erythrocytes in the PNH individual. The first would lack AChE and be complement-sensitive. The second would contain AChE and be normal in their lack of sensitivity to complement. In this study, we demonstrate this contention directly through flow-cytofluorimetric analysis of erythrocyte AChE content at the single-cell level.

MATERIALS AND METHODS

Immunofluorescent labeling of erythrocytes. Whole blood was drawn from normal subjects and PNH patients with EDTA used as an anticoagulant. A 200-μL volume of this blood was washed three times with 500 μL of isotonic phosphate-buffered saline (PBS), pH 7.4. The cells were resuspended in 500 μL of PBS, and 10 μL of AE-2 monoclonal antibody ascites fluid (produced from AE-2 mouse

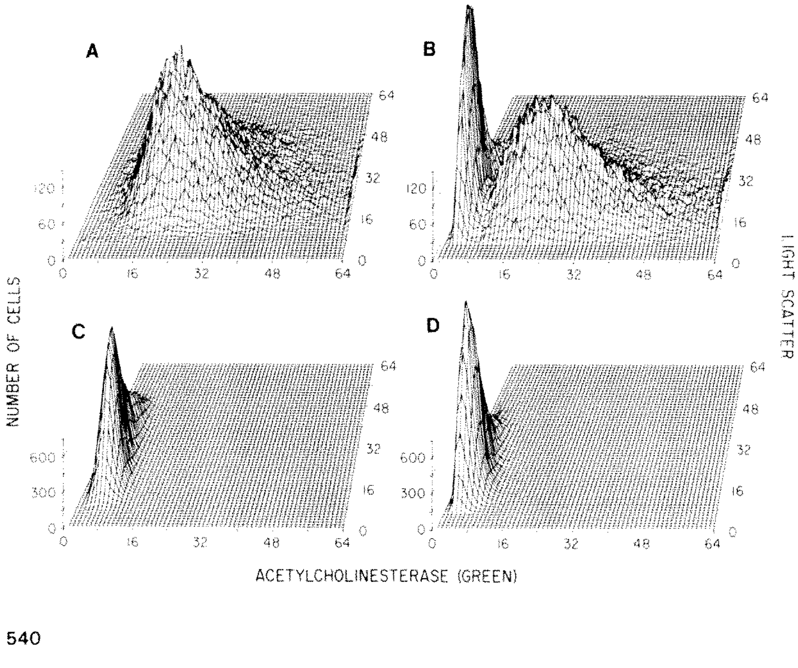

Fig 1. Flow-cytofluorimetric analysis of acetylcholinesterase content on erythrocytes can be used to detect PNH erythrocytes in a PNH patient. Samples were stained immunofluorescently for surface acetylcholinesterase as described in the text. Three-dimensional analysis of acetylcholinesterase (green fluorescence) v relative cell size (light scatter) v cell number is shown. (A) Erythrocytes of a normal individual showing all cells staining positive for acetylcholinesterase. (B) Erythrocytes of a PNH patient demonstrating that a subpopulation of erythrocytes from this patient lack the acetylcholinesterase antigen. (C) and (D) Controls for normal and PNH erythrocytes, respectively, where monoclonal antibody AE-2 was omitted from the labeling procedure.

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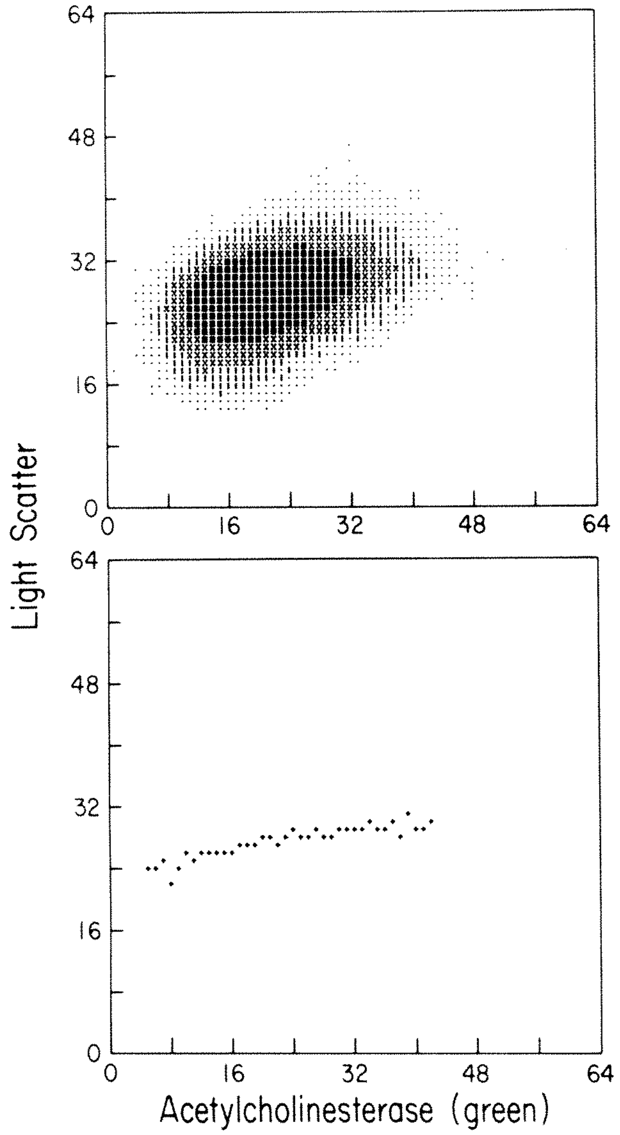

Fig 2. Plots of acetylcholinesterase labeling *v* light scattering of erythrocytes from a normal donor indicate a positive correlation between cell size and AChE content. Contour levels shown in the top panel are set at 5–20, 21–50, 51–150, and greater than 150 cells for the four levels of increasing density in the plot. The same data are shown replotted in the bottom panel as the mean channel of fluorescence for each light-scatter channel.

hybridoma cell line obtained from the American Type Culture Collection, ATCC No. HB-73) was added to the cells.[11] This concentration of ascites fluid was determined to be a saturating dose of primary antibody as determined by separate titration studies. The suspension was allowed to incubate at room temperature for 20 minutes and subsequently washed twice with 500 μL of PBS to remove unbound antibody. Indirect immunofluorescence of the bound immunoglobulin was accomplished by subsequently incubating the erythrocytes with saturating doses (1:100 PBS dilutions) of fluorescein-conjugated goat antimouse IgG (whole molecule, 2.3 mg IgG/mL, F/P molar ratio 4.1) and fluorescein-conjugated rabbit antigoat IgG (whole molecule, 2.7 mg IgG/mL, F/P molar ratio 4.1) (Sigma Chemicals, Inc) for 20 minutes each with two PBS washes after each incubation. Control samples were treated in an identical manner except the first monoclonal antibody was omitted from the labeling protocol. Similar control results were obtained using ascites fluid containing monoclonal antibodies directed against irrelevant proteins yeast cytochrome c oxidase and platelet membrane glycoprotein IIb (data not shown). Where indicated, complement-sensitive cells were first lysed by the sucrose hemolysis test[2] before immunoglobulin labeling.

Flow cytofluorimetry. Labeled erythrocytes were diluted approximately 1:1,000 in PBS immediately before flow-cytofluorimetric analysis. All stained cells were analyzed at approximately 500 cells/s on an EPICS-V flow cytofluorimeter (Coulter Electronics, Inc, Hialeah, Fla) after excitation with a Spectra-Physics 164-05 argon laser, adjusted to deliver 800 mW at 488 nm. Low-angle light scattering was detected through a 2.0 neutral density filter, and green fluorescence was detected at right angles to the incident laser beam through a 510-nm interference and 515-nm bandpass filter combination on the green photomultiplier of the instrument. The light-scatter and fluorescence signals from each cell are collected and stored by the Coulter MDADS integrated microcomputer analysis system in a 64 × 64 matrix array. Routinely, the fluorescence signals measured are gated on a light-scatter profile that precludes contributions from small cell debris and dust.[12] Data collection was set up to stop after 100,000 cells have been analyzed.

RESULTS

Erythrocytes isolated from normal donors and PNH patients were analyzed by flow cytofluorimetry after indirect immunofluorescence labeling of surface AChE with the monoclonal antibody AE-2. Figure 1 displays the three-parameter histogram of light scatter, green fluorescence, and cell number for these cells. Normal erythrocytes all could be shown to have detectable levels of AChE on their surface, as indicated by their positive green fluorescence signal. Figure 2 is a scattergram of the same normal cell sample shown in Fig 1. This alternative presentation of the data shows that in normal individuals, the mean green fluorescence signal increases with increasing light-scatter signal, which indicates a higher level of AChE on the surface of the larger erythrocytes.[12] In contrast, erythrocytes from PNH patients showed two distinct populations of cells with respect to AChE content (Fig 1). The first population has an AChE content and distribution similar to those of the normal erythrocytes. The second population of erythrocytes has undetectable levels of AChE on their surfaces.

Figure 3 shows that cells that are complement-sensitive in the PNH patient lack AChE. Erythrocytes from a normal donor and a PNH patient were independently analyzed for their percentage of PNH cells by complement lysis. The complement lysis test indicated that 19.0% of the hemoglobin in the PNH sample was released. This number was very close to the 19.3% of the erythrocytes detected as AChE-negative by integration of the number of cells under the AChE-negative peak of the PNH cells in Fig 3. There was no hemoglobin release from the normal erythrocytes, and no AChE-negative cells were detected by flow cytofluorimetry. This result suggests that the two analyses were measuring the same cell population. To test this contention further, the remaining PNH (the cells insensitive to complement lysis) were spun down, labeled for AChE, and analyzed by flow cytofluorimetry. Figure 3 clearly indicates that the PNH cells that are complement-lysed lack the surface enzyme

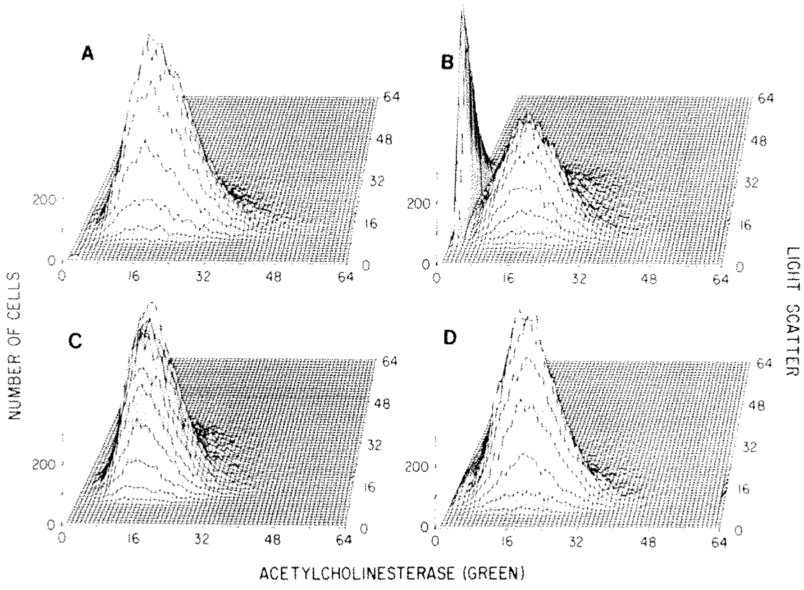

Fig 3. Complement-sensitive cells in PNH patients are AChE-negative cells. Erythrocytes from a normal donor (A) and a PNH patient (B) were analyzed by flow cytofluorimetry for AChE content as in Fig 1. After complement lysis, the unlysed cells were again analyzed by flow cytofluorimetry. Normal erythrocytes had 0% lysis, and the complement-treated cells are shown at C. The PNH patient's cells had 19% lysis, and the cells remaining unlysed after complement treatment are shown at D. These remaining PNH cells had normal levels of AChE.

acetylcholinesterase. Thus, erythrocytes detected as abnormal because they lack surface AChE in PNH individuals are the same set of cells that are complement-sensitive.

DISCUSSION

Current evidence suggests that in PNH, an altered complement pathway is involved in the anemia. Accordingly, much of the recent work on this disease has focused on why the PNH cell is more complement-sensitive than normal cells. Reports suggest that both normal and PNH erythrocytes assemble the alternative pathway C3 convertase in the same fashion. However, the PNH cells activate more C3, resulting in the deposition of increased levels of C3b on the cell surface.[13-15] Since a similar number of activated C3b binds to normal and PNH cells, the greater fixation of C3b on the surface of PNH cells has been attributed to abnormalities on the PNH erythrocyte membrane that favor increased C3 convertase activity. The PNH cells bind the 3Cb covalently to the hydroxyl group of the major erythrocyte sialoglycoprotein (PAS 1, glycophorin A).[15-17] Evidence has also been presented that the sialoglycoproteins of PNH cells may themselves be altered in some PNH patients.[15] Other investigators have suggested that an inhibitor that is involved in regulating the alternate pathway is absent in a subpopulation of PNH erythrocytes.[18,19]

It is clear from these diverse studies that considerable variability may exist among PNH patients with respect to changes in the red cell membrane that ultimately make the cell more complement-sensitive. Accordingly, a current PNH cell classification scheme classifies PNH patients according to their degree of complement sensitivity.[20,21] In some PNH patients, the complement sensitivity is very close to that of normal individuals.[22] This fact, in combination with the variability of complement potency of donor sera used for hemolysis tests and the low percentage of complement-sensitive cells in some PNH patients, makes positive diagnosis of PNH difficult in some patients.[23]

In this study, we have shown that the ectoenzyme acetylcholinesterase is absent in the complement-sensitive clones of cells present in a PNH patient. Although the enzyme does not appear to play any role in the disease state, it may present a clearer criterion for the diseased cells than complement sensitivity. We have therefore developed a method for the detection of acetylcholinesterase at the single-cell level. To date, over 50 blood samples from seven previously diagnosed PNH patients and over 100 normal samples have been analyzed by this method. In all PNH cases, a distinct population of AChE-negative cells is discernible in addition to cells with the normal content of AChE. All normal samples have lacked this AChE-negative population of cells.

This method has distinct advantages over current PNH diagnosis procedures: It is quantitative for the percentage of abnormal cells circulating in the PNH patient, it uses a monoclonal antibody that is readily available as a standardized diagnostic reagent, it requires a very small blood volume, and it is capable of highly accurate and reproducible determinations even on samples with very low percentages of abnormal cells. Although it does require a flow cytofluorimeter for quantitative measurements, qualitative analyses can be performed on a fluorescence microscope. Finally, the method allows us not only to determine quantitatively the percentage of abnormal erythrocytes but also to correlate the acetylcholinesterase content of a cell with any other parameter detectable by flow cytofluorimetry. In this study, we have also noted a positive correlation between erythrocyte size and acetylcholinesterase content. This is in contrast to previous studies, which indicate that there is no correlation between another erythrocyte membrane component, protein 3, and cell size.[12] Such results can be explained by proposing a selective loss of membrane material during erythrocyte aging. The potential of multiparameter analysis of erythrocytes in PNH is currently under active investigation.

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CONCISE REPORT

Enhanced Survival of Sickle Erythrocytes Upon Treatment With Glyceraldehyde

By Lennette J. Benjamin and James M. Manning

Glyceraldehyde has been demonstrated to be an antisickling agent in vitro. In the present investigation, chromium-51 red cell studies were used to investigate the life span in vivo of sickle erythrocytes after treatment with glyceraldehyde in vitro. The mean survival ($T_{1/2}$) of control cells was 5.8 ± 1.6 days, whereas cells treated with 10 mmol/L or 20 mmol/L glyceraldehyde survived 9.0 ± 1.4 ($P < .05$) and 11.3 ± 0.8 ($P < .002$) days, respectively. The extent of modification by glyceraldehyde was 0.4 to 1.0 lysine residue per hemoglobin tetramer. These studies demonstrate not only a prolongation of the life span of sickle erythrocytes by treatment with glyceraldehyde but also the absence of any deleterious effects that would be revealed by this study.

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GLYCERALDEHYDE has been shown to have significant antisickling activity in vitro through its ability to modify hemoglobin S.[1-3] This modification occurs as a result of the reaction between the aldehyde portion of the molecule and some of the amino groups of hemoglobin S leading to Schiff base formation and subsequent Amadori rearrangement to a stable ketoamine[4] (Fig 1).

The major sites of the reaction were found to be Lys-16(α), Val-1(β), Lys-82(β), Lys-59(β), and Lys-120(β).[4] The major effect of this reaction is a reduction in the ability of deoxyhemoglobin S to polymerize, due mainly to the modification at Lys-16(α).[5] The modification has a slight effect on the oxygen affinity of hemoglobin at low concentrations of glyceraldehyde (5 mmol/L and 10 mmol/L).[1,5] However, at higher concentrations of the compound (20 mmol/L), there is a measurable decrease in the P_{50}.[2,6] The stability of the ketoamine adduct suggests that glyceraldehyde has a suitable half-life for therapeutic use.[7,8] However, prior to performing pharmacologic and toxicity studies and subsequent therapeutic trials, such a drug should be demonstrated to be nontoxic to sickle erythrocytes. Earlier studies in vitro indicated that even though there was a discernible amount of reaction of glyceraldehyde with red cell membrane proteins, there was no adverse effect on either the osmotic fragility or the filterability of the cells[6] or on the activity of red cell enzymes.[7] Other indications that the red cell was not compromised were the findings that both the high viscosity[6] and the elevated cation permeability[7] of sickle cells were decreased.

The study reported here was designed to determine whether in vitro incubation of sickle erythrocytes with glyceraldehyde was attended by a beneficial or a deleterious effect on their survival in vivo.

MATERIALS AND METHODS

Patients. Five patients with homozygous sickle cell anemia were studied at Rockefeller University Hospital during the asymptomatic or steady-state period. The study protocols were approved by the institutional review board for human studies. The patients gave informed consent and were admitted as inpatients during the studies.

Red cell survival studies. Red cell survival studies were performed using chromium-51 according to the method of Grey and Sterling.[9] An initial control study was performed on whole blood that was incubated with sterile normal saline for 1½ hours at room temperature. After removal of the plasma, the cells were washed twice with sterile normal saline and labeled with 100 μCi of ^{51}Cr. Unreacted radiolabel was removed by washing, and the cells were then suspended in sterile normal saline and infused into the patient. A blood sample was obtained 24 hours later (day 0) and each day thereafter until $T_{1/2}$ (50% of the initial radioactivity on day 0) was reached. After the disappearance of this initial dosage of radioactivity, the drug phase of the study was performed, using the identical procedure as in the control study except that whole blood was incubated for 1½ hours at room temperature with 10 mmol/L or 20 mmol/L glyceraldehyde dissolved in sterile normal saline. After removal of plasma, the cells were washed, labeled with ^{51}Cr, washed, and infused into the patient as described.

Determination of hemoglobin modification. The extent of modification of hemoglobin S was analyzed on an aliquot of the protein that was reduced with sodium borohydride at pH 6 and then subjected to acid hydrolysis.[1] By the reduction treatment, lysine residues that have reacted with glyceraldehyde yield a dihydroxypropyllysine derivative that is stable to acid hydrolysis in 6N HCl. This derivative is eluted from the 0.6 × 10 cm column of the amino acid analyzer in a position different from other amino acids in the hydrolyzate.[1] The amount of reaction with glyceraldehyde is expressed as the ratio of dihydroxypropyllysine to the amount of hemoglobin tetramer.

Data analysis. Red cell survival studies were analyzed using the Prophet Computer Network (NIH Division of Research Resources). Cell survival was plotted as log % radioactive counts *v* days using the straight line of the best fit through the experimental points. Comparisons of the mean $T_{1/2}$ values of control and treated groups were performed using *t* tests. The paired *t* test was used for analysis of the difference in $T_{1/2}$ of the control and treated cells for each patient.

RESULTS

Patients. Five patients (three males and two females), aged 25–39, with hemoglobin S were studied (Table 1). The degree of anemia ranged from 6.5 to 8.5 g Hb. None of the patients had a palpable spleen.

Red cell survival studies. The erythrocytes of all patients showed an increase in their mean survival time after treatment with glyceraldehyde compared with the mean survival time of buffer-treated autologous control cells (Fig 2). The mean survival ($T_{1/2}$) of control cells was 5.8 ±

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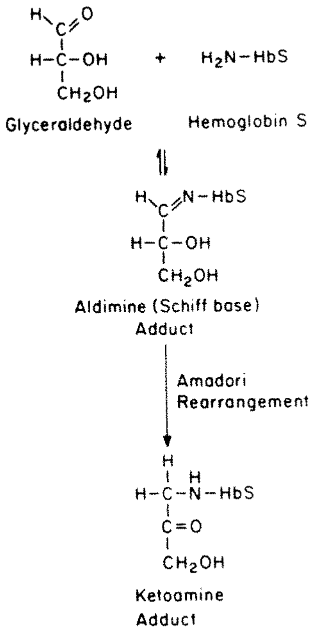

Fig 1. Reaction of glyceraldehyde with hemoglobin S.

1.6 days, whereas cells treated with 10 mmol/L and 20 mmol/L glyceraldehyde survived 9.0 ± 1.4 ($P < .05$) and 11.3 ± 0.8 days ($P < .002$), respectively. Paired *t*-test analysis of the $T_{1/2}$ values also indicated statistically significant improvement ($P < .002$) in the survival of the treated cells. In a separate study in vitro, we found that the ^{51}Cr elution profile was not affected by treatment with glyceraldehyde.

Modification of hemoglobin. The extent of the reaction of glyceraldehyde was determined after reducing the ketoamine adduct with sodium borohydride. Upon acid hydrolysis, 0.4 to 0.5 dihydroxypropyllysine groups per hemoglobin tetramer were found at the 10 mmol/L concentrations and 0.8 to 1.0 groups incorporated at the 20 mmol/L concentrations.

DISCUSSION

These studies demonstrate a prolongation of the life span of sickle erythrocytes after treatment with glyceraldehyde. The mean $T_{1/2}$ was increased significantly by 10 mmol/L and 20 mmol/L glyceraldehyde. The same conclusion was reached in comparing the results for each patient by a paired *t* test. In three of the five patients studied, the $T_{1/2}$ was increased at least twofold. These studies demonstrate not only a beneficial effect on sickle erythrocytes but also that glyceraldehyde in the concentrations used is not toxic to sickle erythrocytes.

Table 1. Baseline Hematologic Data on Patients

Patient	Age	Sex	Hb (g/dL)	Hematocrit (%)	Red Cells (× 10^6 cells/mm^3)	Final Concentration of Glyceraldehyde (mmol/L)
A 1*	25	M	7.7	23.8	2.96	0
2*			8.2	23.8	3.10	10
B 1	39	M	7.3	22.3	2.85	0
2			6.5	20.2	3.51	10
C 1	37	F	7.4	21.2	2.09	0
2			7.6	21.2	2.38	20
D 1	25	M	8.5	23.4	3.02	0
2			8.5	23.5	3.00	20
E 1	26	F	7.1	20.6	2.37	0
2			6.5	20.3	2.55	20

*1, initial control study; 2, glyceraldehyde-treated red cells.

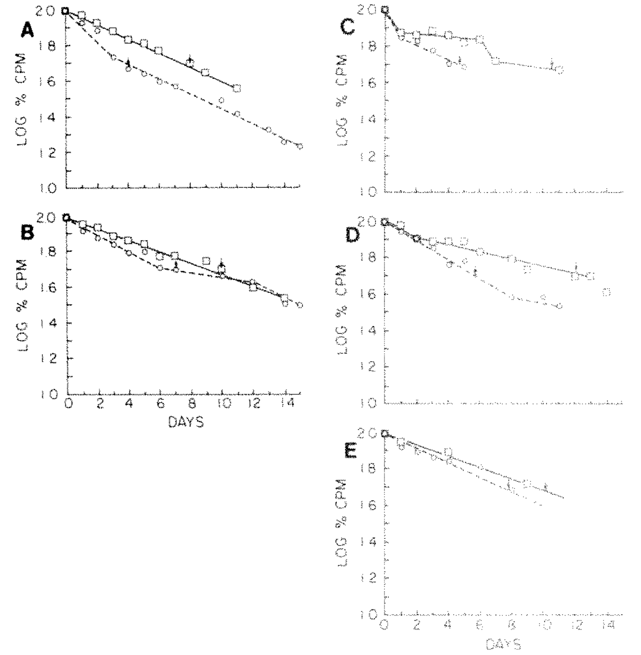

Fig 2. Chromium-51 red cell survival studies of treated and untreated erythrocytes. Cell survival is plotted as log % counts v days using the straight line of the best fit (see text). The left panel shows the survival profiles of the red cells from two patients whose erythrocytes were untreated (○) or treated with 10 mmol/L glyceraldehyde (□) as described in the text. The right panel shows the profiles of control cells (○) and cells treated with 20 mmol/L glyceraldehyde (□). The mean survival times ($T_{1/2}$), as indicated by the arrows, are as follows: patient A, 4.0 days (untreated), 8.0 days (treated); patient B, 7.0 days (untreated), 10.0 days (treated); patient C, 4.7 days (untreated), 10.6 days (treated); patient D, 5.5 days (untreated), 12.2 days (treated); patient E, 7.8 days (untreated), 11.2 days (treated).

Glyceraldehyde has been shown to have the properties that a compound should possess before performing toxicity and pharmacology studies and prior to consideration for clinical trials: (1) It has been shown to have significant antisickling effects in vitro by several criteria in different laboratories,[1-3,6] (2) its mechanism of action is established,[5] (3) it has been shown to have no severe adverse effects on red blood cell function and metabolism,[6,7] and (4) as demonstrated in this study, it has a beneficial effect on erythrocyte survival time. Thus the slight degree of crosslinking of membrane proteins observed at high concentrations of glyceraldehyde in vitro[6] is apparently of little consequence in vivo, at least as ascertained by these initial cell survival studies.

The pharmacologic literature on glyceraldehyde is fairly extensive in animal studies where it has been evaluated as an antileukemia agent.[10-12] In one such study, it was determined that glyceraldehyde led to a decrease in the incorporation of labeled amino acids into proteins.[12] This result might have implications regarding future toxicity. However, of the hemoglobin-modifying agents presently being considered for treatment of sickle cell anemia, glyceraldehyde is estimated by toxic-to-therapeutic ratios to be one of the least toxic.[13] It has an LD_{50} of approximately 3 g/kg when injected intraperitoneally into mice.[10]

Although these findings are encouraging, extensive evaluation needs to be performed regarding the toxicity of glyceraldehyde before clinical trials can be carried out via the oral or parenteral route. The results described here and in previous studies, which show significant antisickling activity, suggest the feasibility of limited clinical testing of glyceraldehyde via the extracorporeal route. This approach would permit the testing of a larger population of cells and thereby address the question of whether there is a selective sparing of deoxygenation of a small aliquot of cells such as used in the present study. Investigations to extend these studies using a double isotope cohort label and to define the optimal reaction conditions are in progress. This information will permit an evaluation not only of the efficacy of this agent but also of potential problems regarding antigenicity and any adventitious reactions with macromolecules other than hemoglobin S.

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CONCISE REPORT

A Novel Mutation in the TATA Box in a Japanese Patient With β^+-Thalassemia

By Yoshihiro Takihara, Takanori Nakamura, Hideo Yamada, Yasuyuki Takagi, and Yasuyuki Fukumaki

A single base substitution (A-G) at position −31 within the highly conserved proximal promoter element, the TATA box, was identified in the β-globin gene cloned from a Japanese woman with β^+-thalassemia. It appears that she is homozygous for this specific allele, as determined by haplotype analysis using seven different polymorphic sites in the β-globin gene cluster. Transient expression of the mutant gene in COS cells revealed a 45% reduction in β-globin RNA production, relative to normal. These results establish the functional significance of the second base of the TATA box for in vivo transcription of the human β-globin gene.

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BETA-THALASSEMIAS ARE heterogeneous inherited disorders characterized by defective synthesis of the β-globin chains of adult hemoglobin. Two types, β^0- and β^+-thalassemia, have been defined. In patients with homozygous β^0-thalassemia, there are no β-globin chains, whereas β-globin chains are produced at low levels in patients with homozygous β^+-thalassemia.[1] Molecular defects have been defined for many β-thalassemias, including gene deletion, as well as point mutations that cause abnormalities in RNA transcription, RNA processing, or premature termination of translation.[2,3] Study of these mutations has provided insight into various aspects of expression of the β-globin gene.

Each ethnic group studied to date has been shown to have several distinct mutations. Characterization of mutations in additional ethnic groups should lead to identification of new mutations and provide additional data on the genetic heterogeneity of single-gene diseases. We now report a novel TATA box mutation in the β-globin gene of a Japanese woman homozygous for β^+-thalassemia. Studies on the expression of the β-thalassemia gene in heterologous cells showed that the thalassemia phenotype was the result of inefficient transcription.

MATERIALS AND METHODS

Patient. The patient studied was a Japanese woman with transfusion-independent β-thalassemia intermedia. Splenomegaly and jaundice were noted. Hematologic data were Hb 7.2 g/dL, hematocrit 32%, RBC 430 × $10^4/\mu L$, mean corpuscular volume (MCV) 74.4 μm^3, mean corpuscular hemoglobin (MCH) 16.7 pg, mean corpuscular hemoglobin concentration (MCHC) 22.5 g/dL, and reticulocyte count 3.6%. There was poikilocytosis with target cells and basophilic stippling on the peripheral blood smear. Bone marrow examination revealed erythroid hyperplasia; the granuloid-to-erythroid cell ratio was 0.21. Hemoglobin electrophoresis on cellulose acetate revealed HbA at 80.1% and HbA_2 at 5.9%. The HbF level was estimated to be 14.0% by the alkali denaturation method.[4] The ratio of β- to α-globin chain synthesis in the peripheral erythroid cells was 0.34. The patient was the product of a consanguineous marriage. Hemoglobin analyses of both parents showed HbA_2 levels of 4.6% and 4.9%, respectively, and HbF levels of 0.6% and 1.2%.

DNA polymorphism haplotype analysis. High-mol-wt DNA was prepared as described.[5] Restriction site polymorphisms in the β-globin gene cluster were determined by Southern blot analysis using appropriate probes.[6,7]

Gene cloning and DNA sequencing. The β-thalassemia gene was cloned in bacteriophage Charon 28 as a 7.8-kilobase (kb) *Hind*III fragment. The β-globin gene region was subcloned as a 4.9-kb *Bgl*II fragment in pBR322 and sequenced by the method of Maxam and Gilbert.[8]

Construction of the globin-SV40 recombinant molecules. The plasmid vector pSVori2172, which contains the SV40 *Pvu*II-*Bgl*II fragment with the origin of DNA replication and tandemly repeated 72-base-pair sequence, was used as a vector for expression of the globin genes in COS cells (Fig 1). The construction of this SV40 vector has been described in detail.[9]

Globin gene expression in COS cells. COS cells were grown in Dulbecco's modified Eagle's medium with 10% newborn calf serum. Twenty micrograms of the β-globin gene recombinant and 2 μg of an α-globin gene recombinant[10] (an internal reference) were added as calcium phosphate precipitates to each 10-cm culture dish of subconfluent COS cells.[11] Total cellular RNA was prepared from the transfected cells after incubation for 48 hours.[12]

SI nuclease mapping. Probe excess-SI nuclease mapping of the total cellular RNA was performed using appropriate probes labeled at their 5′ termini[13] (see Fig 4). Hybridizations were carried out overnight at 51 °C. Hybrids were digested at 37 °C for 30 minutes with 7–10 U/mL SI nuclease purchased from Sankyo Company, Ltd. Protected DNA fragments were electrophoresed in urea-polyacrylamide gels and autoradiographed with intensification. The amount of β-globin RNA was standardized for the difference in the α-globin RNA signals on comparison of the normal and thalassemia β-globin gene.

RESULTS

DNA polymorphism haplotype of the β-globin gene cluster. To determine whether the patient was homozygous for a thalassemia allele, we analyzed her DNA with respect to common DNA polymorphisms within the β-globin gene cluster. This patient was homozygous at seven different polymorphic restriction sites, as shown in Fig 2. Homozygosity for one haplotype and the consanguineous marriage of her parents strongly indicated that the patient was homozygous for a particular type of the β-globin gene mutation. Thus we used a single cloned β-globin gene for further analysis.

DNA sequence of the β-thalassemia gene. We determined the DNA sequence of the thalassemia gene region

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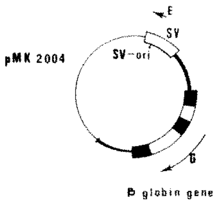

Fig 1. Structure of the SV40 recombinant containing the normal and the mutant globin gene. Construction of the recombinants is described in the text. SV, the SV40 *PvuII-BglII* fragment containing sequences necessary for efficient β-globin gene expression in COS cells; SV-ori; replication origin of the SV40 DNA; β-globin gene, a 4.9-kb *BglII* fragment containing the β-globin gene. Polarity of SV40 early transcription unit and β-globin gene transcription unit is indicated by arrows E and G, respectively.

from 200 bases 5′ to the Cap site to 200 bases 3′ to the polyadenylation site of the cloned β-globin gene. Comparison of the nucleotide sequence of the thalassemia β-globin gene to that of a human β-globin gene[14] revealed two base changes, an A-G change at position −31 5′ to the Cap site and a G-T change at position 74 in IVS2 (Fig 3). The A-G substitution is located within the TATA box, also called the Goldberg-Hogness box (CATAAAA to CGTAAAA). The TATA box is known as a conserved sequence that is required for efficient and accurate transcription of many eukaryotic protein-coding genes.[15] Thus the A-G transition found in the globin gene of the patient could cause a thalassemia phenotype by decreasing the level of the transcription of the gene. The G-T substitution in IVS2 has been reported to be a DNA polymorphism also found in normal globin genes.[7,14]

Functional analysis of the β-thalassemia gene. To investigate the possibility that the A-G substitution at position −31 was responsible for the reduction in β-globin chain production, we examined the function of the gene in a transient expression assay in COS cells. Plasmid recombinants containing the normal and thalassemia β-globin genes were transfected into COS cells with an α-globin gene recombinant as an internal standard. Total cellular RNA was extracted from COS cells after incubation for 48 hours and then analyzed by S1 nuclease mapping after hybridization with the β- and α-globin gene probes (Fig 4). The major S1 nuclease–resistant band derived from β-globin RNA was two nucleotides longer than expected (Fig 4) because the two 3′ terminal nucleotides of exon 1 and IVS1 are identical. The major band generated by α-globin RNA and the minor band by β-globin RNA were due to the partial failure of S1 nuclease to remove the GC-rich segment at the 3′ end of IVS1 as observed by Busslinger et al.[16] The mutant globin gene directed the synthesis of the β-globin RNA to a lesser extent than normal (Fig 4). Direct scanning of the autoradiogram demonstrated a 45% reduction of β-globin RNA relative to normal. When we performed the S1 nuclease mapping experiment with the 5′ part of the β-globin probe (a 344-nucleotide-long *Hin*fI fragment), no differences between the mutant and the normal genes were observed at the initiation site of transcription (data not shown).

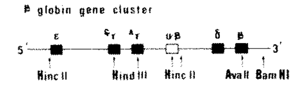

Fig 2. Haplotype of the β-globin gene cluster. The seven polymorphic restriction sites are shown in the β-globin gene cluster from a Japanese woman with β-thalassemia; + and − indicate the presence or absence of a site, respectively.

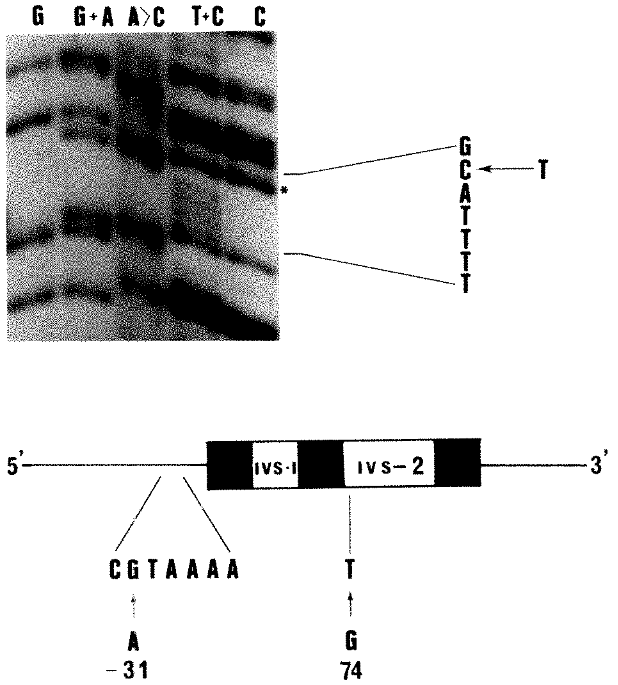

Fig 3. DNA sequence of the β-globin gene of the Japanese patient and the autoradiograph of the gel demonstrating the sequence of the sense strand in the vicinity of the TATA box. The A-G substitution at position −31 to the Cap site and another base substitution that was considered to be a DNA sequence polymorphism are shown.

DISCUSSION

We observed that the one base substitution (A-G) at position −31 (the second base of the TATA box) in the β-globin gene isolated from a Japanese woman with β^+-thalassemia reduced the efficiency of transcription by a factor of 2. Three other types of mutation in the TATA box of the β-thalassemia globin genes have been described.[17-19] The first detection was in a Kurdish Jew with an A-C change at position −28.[17] This mutant gene directed the production of a two- to threefold decrease in transient expression in Hela cells and a fourfold reduction in in vitro transcription assays.[18] The second type of mutation was an A-G substitution at the same position[19] detected in a Chinese patient. A three- to fivefold decrease in transcriptional efficiency of this TATA box mutant was seen using transient expression in Hela cells. In RNA prepared from the erythroid cells of the patient, β-globin RNA was one-tenth as abundant, relative to α-globin RNA, than normal. An A-G transition at position −29 was recently detected in an American black.[20] This mutant gene produced β-globin RNA at 25% the normal level both in heterologous and erythroid cells. These results suggest that expression of the β-globin gene with a one-base

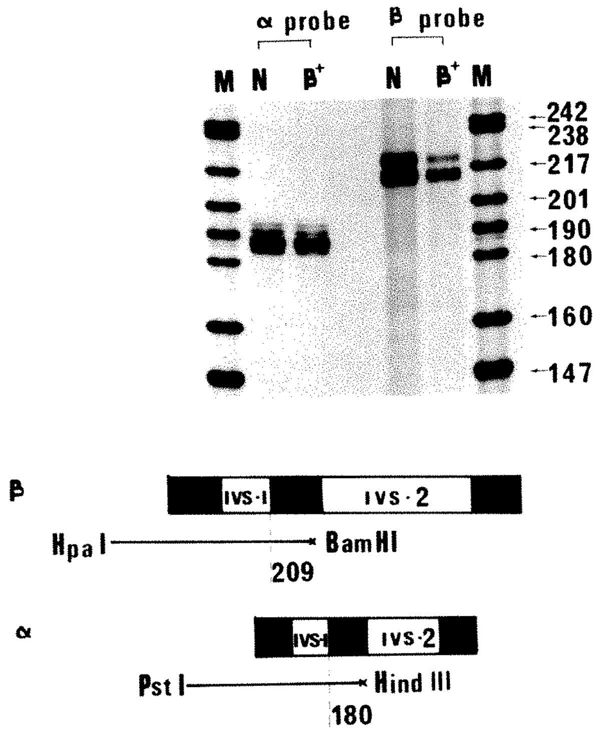

Fig 4. Expression of the −31 A-G mutant gene in COS cells. Normal and mutant β-globin gene–SV40 recombinants were separately cotransfected with normal α-globin gene–SV40 recombinants into COS cells. SI nuclease mapping was performed using the indicated 5′-labeled DNA probes.

substitution within the TATA box in heterologous cells reflects the phenotype attributed to the mutation in patients. In the case of the thalassemic gene we studied, the deficiency of β-globin RNA in heterologous cells was comparable to that observed in erythroid cells. The ratio of β- to α-globin chain biosynthesis was 0.34 in peripheral erythroid cells of this patient, who is homozygous for the mutant allele. Thus the TATA box mutation in the β-globin gene we studied causes a modest reduction in β-globin RNA production and leads to a mild thalassemia phenotype in the homozygous patient. The two mutations at positions −87 and −88, which lie in the sequence ACACCC, proposed as a distal promoter element,[21] also have a quantitatively similar effect on the transcriptional efficiency in β-thalassemia patients.[22,23]

The TATA box is important not only for the efficiency of transcription but also for accuracy in the transcription start site.[16] However, the three types of TATA box mutations of β-globin genes mentioned (A-G at position −28, A-G at position −29, and A-G at position −31) have only a quantitative effect on gene expression. Dierks et al[21] observed little or no heterogeneity in the initiation site of the transcript when the TATA box of the rabbit β-globin gene was deleted but a longer stretch of sequence preceding the Cap site was preserved. These observations suggest that the transcription initiation site of the β-globin gene are not affected by TATA box mutations shown in thalassemic genes because the sequence from the Cap site to the TATA box was preserved.

The TATA box mutation studied here most likely arose primarily in a Japanese person, since the ancestors of this patient are Japanese and this same type of mutation has not been found among other ethnic groups. This is the first report of the molecular basis of β-thalassemia in a Japanese person.

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CONCISE REPORT

Concordance of a Point Mutation 5′ to the $^{A}\gamma$-Globin Gene With $^{A}\gamma\beta^{+}$ Hereditary Persistence of Fetal Hemoglobin in Greeks

By Pamela G. Waber, Michael A. Bender, Richard E. Gelinas, Christos Kattamis, Aris Karaklis, Koula Sofroniadou,
George Stamatoyannopoulos, Francis S. Collins, Bernard G. Forget, and Haig H. Kazazian, Jr.

In the Greek $^{A}\gamma\beta^{+}$ type of hereditary persistence of fetal hemoglobin (HPFH), adult heterozygotes produce about 20% fetal hemoglobin (HbF), which is predominantly of the $^{A}\gamma$ chain variety. The affected β-globin gene cluster produces near normal amounts of β-like globin, but in a $^{A}\gamma$ to β ratio of 20:80 instead of 0.5:99.5. Gelinas et al and Collins et al have shown a G to A change 117 nucleotides 5′ to the $^{A}\gamma$ gene in two Greeks with $^{A}\gamma\beta^{+}$ HPFH. To demonstrate that this change is not a neutral polymorphism, we carried out hybridization with oligonucleotide probes (19mers) specific for the normal and the mutant sequences. While normal probe identified the $^{A}\gamma$ fragment in genomic DNA of all subjects studied, mutant probe was positive only in Greeks with $^{A}\gamma\beta^{+}$ HPFH. In sum, 108 β-globin gene clusters of individuals without HPFH were negative when tested with mutant probe, but all 11 affected individuals of six families with Greek $^{A}\gamma\beta^{+}$ HPFH (two previously sequenced and four new families) were positive with mutant probe. These data support the conclusion that the −117 mutation is causative of $^{A}\gamma\beta^{+}$ HPFH in Greeks.

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HEREDITARY persistence of fetal hemoglobin (HPFH) is a clinically benign condition that is genetically heterogeneous. It is characterized by increased amounts of fetal hemoglobin (HbF) in adult life in the absence of erythropoietic stress.[1,2] Since mutations that produce HPFH affect the normal program of fetal to adult globin-switching, they are of considerable interest. Knowledge of the nature of such mutations should provide insight into control of gene expression and could be of clinical benefit to individuals with β-globin disorders if the switching process could be reversed.

Most individuals with HPFH carry large deletions of the β-globin gene cluster, but the size of these deletions and their location has made it difficult to discern subtle features of the switch mechanism.[3–7] Recently two groups of investigators have suggested that the DNA sequences which reside 3′ to the γ-globin genes as a consequence of the deletion are characterized by chromatin in an open configuration, which allows continued transcription of the γ-globin genes 5′ to them[8] (J.T. Elder and M.T. Groudine, personal communication, June 1985).

However, because of difficulty in discovering the true relationship of the deletions to continued gene expression in cis, recent emphasis has been placed on characterization of the nondeletion types of HPFH. Three different base substitutions in the promoter regions of the γ-globin genes have been reported in these conditions. A C → G change at position −202 was found in the black $^{G}\gamma\beta^{+}$ HPFH in which heterozygotes produce 15% of 25% HbF, almost exclusively of the $^{G}\gamma$ type.[9] A C → T change at position −196 is present in Italians with $^{A}\gamma\beta^{+}$ HPFH which is similar to the HPFH mentioned above except that the HbF is of the $^{A}\gamma$ type.[10] The third putative mutation is a G → A substitution 117 nucleotides 5′ to the $^{A}\gamma$ gene in Greek $^{A}\gamma\beta^{+}$ HPFH.[11,12] In this latter condition, which is the predominant type of HPFH in Greeks,[13,14] heterozygotes make about 20% HbF, most of which is of the $^{A}\gamma$ chain variety. In this condition, like other HPFH conditions of the nondeletion type, the affected β-globin gene cluster produces near normal amounts of β-like globin, but in a $^{A}\gamma$ to β ratio of 20:80 instead of the normal 0.5:99.5.

In order to document the causative nature of the above-mentioned changes 202, 196, and 117 nucleotides 5′ to the cap site of the affected genes, demonstration of abnormal behavior of the cloned gene in gene transfer or transgenic systems is required. To date, such proof has not been obtained.

Therefore, we have attempted to strengthen the evidence for the causative nature of these mutations by studying the concordance of the mutations with the HPFH conditions. First, we showed that the −202 mutation is not present in normal Blacks who lack the $^{G}\gamma\beta^{+}$ HPFH condition.[15] This analysis was facilitated by the fact that the −202 mutation eliminated an Apa I restriction site. Now we have studied 11 affected individuals of six families with Greek $^{A}\gamma\beta^{+}$ HPFH (two previously sequenced and four new families), and 108 normal and thalassemic β genes in Greeks, Italians, Asian Indians, and others. Because the −117 mutation does not alter a restriction site, oligonucleotide hybridization of genomic DNA was used. In this study we found complete concordance between the G → A change at −117 and the Greek $^{A}\gamma\beta^{+}$ HPFH, thereby strengthening the argument that this mutation is the cause of the clinical phenotype.

MATERIALS AND METHODS

Subjects. DNA was obtained from Greek, Italian, and Asian Indian couples, referred for prenatal diagnosis of β-thalassemia, and

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From the Genetics Unit, Department of Pediatrics, the Johns Hopkins University School of Medicine, Baltimore, Md, the University of Washington School of Medicine, Seattle, Agia Sophia Childrens Hospital, Guodi, Athens, Greece, the National Blood Transfusion Center, Nikea, Pireus, Greece, the Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, Mich, and the Hematology Section, Department of Medicine, Yale University School of Medicine, New Haven, Conn.

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their offspring and from 11 Greek individuals of six families with $^{A}\gamma\beta^{+}$ HPFH. Two of these 11 individuals were those in whom the −117 mutation was first characterized.

Oligonucleotide hybridization. For oligonucleotide hybridization 10 μg of genomic DNA were digested with 30 units of the restriction endonuclease Xho I for 15 hours at 37 °C and subjected to electrophoresis in a 1% agarose gel.

Oligonucleotides consisting of 19 nucleotides and representing either normal or mutant sequences were synthesized on an Applied Biosystems DNA synthesizer. The mutant sequence used was CAGCCTTGCCTT$\boxed{\text{A}}$ACCAAT with the boxed A replacing the normal G at this position. The normal oligomer used was the complementary sequence to that shown with normal C at position −117. Because the sequence chosen for analysis includes a CCAAT homology region, it was expected that the oligonucleotides produced might hybridize with a large number of sequences in the human genome. Thus, we used a restriction endonuclease, Xho I, which cuts the bulk of the genome into very large fragments (>20 kb), while the $^{A}\gamma$ gene is present in a 4.9 kb fragment.

Oligonucleotides were end-labeled using T_4-polynucleotide kinase and ^{32}P-ATP. After labeling, the specific activity of the oligonucleotide was maximized by separating ^{32}P-labeled oligomer from unlabeled oligomer by electrophoresis on a 20% polyacrylamide sequencing gel. The gel region containing labeled oligomer was excised and the oligomer was extracted by the crush and soak method.[16] Hybridization of labeled oligomer with the dried gel was carried out as described at Tm minus 4 °C for three hours.[16] The gel was washed in 2XSSPE/0.1% sodium dodecyl sulfate (SDS), and the final wash in 2XSSPE was 1° to 2° below the calculated Tm for two to three minutes. 1XSSPE is 10 mmol/L sodium phosphate, 0.18 mol/L sodium chloride, 1 mmol/L EDTA, and pH 7.4. Gels were then autoradiographed as described.[16] Gels hybridized to probe in this manner could be reused with new probe after they were stripped by soaking in denaturing buffer for half an hour and neutralizing buffer for half an hour.

RESULTS

In order to demonstrate concordance of the −117 mutation and the $^{A}\gamma\beta^{+}$ HPFH phenotype in Greeks, it was important to study a number of Greeks who had the $^{A}\gamma\beta^{+}$ phenotype along with Greeks and Italians who lacked the phenotype.

DNA of 11 individuals with $^{A}\gamma\beta^{+}$ HPFH representing six different families was hybridized with the mutant and normal oligomer probes (Fig 1). All 11 of these individuals demonstrated a band at 4.9 kb with both mutant and normal probe. In one instructive family, the affected mother and her two affected children were positive with both probes, while the normal father showed hybridization with the normal probe only.

In one of the Greek families with $^{A}\gamma\beta^{+}$ HPFH, DNA from one child that was positive for hybridization with both probes was further analyzed by genomic cloning and DNA sequence analysis. The sequence of the $^{A}\gamma$ gene promoter region was determined for five independently isolated clones: two sequences were normal and three showed the G → A change at position −117, thus confirming the results obtained by the oligonucleotide hybridization assay.

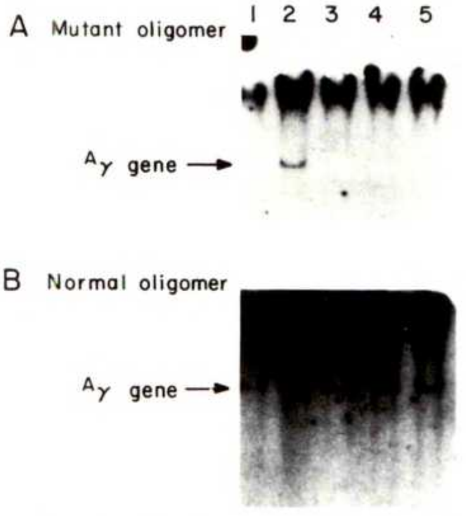

Fig 1. Oligonucleotide hybridization with probes specific for sequences 111–129 nucleotides 5′ to the $^{A}\gamma$ globin gene. Normal oligomer (B) has a C at position −117 while mutant oligomer (A) has an A at position −117 (see Methods). Ten micrograms DNA of five individuals including one Greek with $^{A}\gamma\beta^{+}$ HPFH (lane 2) was digested with Xho I and electrophoresed in a 1% agarose gel. The gel was then hybridized with mutant oligomer (A). The $^{A}\gamma$-globin gene is contained in a 4.9 kb Xho I fragment (arrow). After autoradiography, the gel was stripped of hybridizing probe and reannealed with normal oligomer (B). The individual in lane 2 demonstrates heterozygosity for the mutant sequence, while those in lanes 1, 3, 4, and 5 are homozygous for the normal sequence.

A total of 28 β^{A} and 27 β^{thal} alleles from Greek individuals were analyzed with both probes (Table 1). All individuals tested who lacked the $^{A}\gamma\beta^{+}$ HPFH phenotype were positive with the normal probe only. Seventeen of the chromosomes tested had the same haplotype (I) as that previously observed associated with $^{A}\gamma\beta^{+}$ HPFH.[11,12,17] Haplotype determination has not been completed on the four new families with $^{A}\gamma\beta^{+}$ HPFH reported here. In addition, 13 β^{A} and 16 β^{thal} alleles in Italians, ten β^{A} and eight β^{thal} alleles in Asian Indians, and six alleles of other ethnic groups were tested, and all of these

Table 1. β-globin Gene Clusters Lacking $^{A}\gamma\beta^{+}$ HPFH and Negative for the −117 Mutation Adjacent to the $^{A}\gamma$-Globin Gene by Oligonucleotide Hybridization

	Greeks		Italians		Indians		Other*	
Haplotype§	β^{A}	β^{thal}	β^{A}	β^{thal}	β^{A}	β^{thal}	β^{A}	β^{thal}
I	17	13	12	8	9	3†	3	—
II	1	3	—	5	—	—	—	—
III	2	1	—	—	—	1	—	—
V	1	8	—	1	1	—	1	1
VII	1	1	1	1	—	3	—	—
IX	3	—	—	1	—	1	1	—
Unknown	3	1‡	—	—	—	—	—	—
Totals	28	27	13	16	10	8	5	1

*Among these six chromosomes, three are in blacks, two in Europeans, and one in a Chinese.

†One chromosome contains a β^{0} globin gene.

‡$\delta\beta$ Thalassemia chromosome with deletion of δ and β genes.

§As defined in reference 17.

gave a negative result with the mutant probe and a positive result with the normal probe.

DISCUSSION

HPFH syndromes are of great importance because they provide an opportunity to discover cis-acting DNA sequences important in developmental regulation. Cloning and sequence analysis of the Greek HPFH, which is predominantly of the $^{A}\gamma\beta^{+}$ type, has shown a mutation of G to A 117 nucleotides 5′ to the cap site of the $^{A}\gamma$ gene.[11,12] However, because studies that demonstrate abnormal regulation of this gene in gene transfer systems or transgenic animals have not yet been definitive, it is still not proven whether the mutation is actually responsible for the phenotype, or is merely a polymorphic variation of no functional consequence which is closely linked to the mutant gene. Here we have asked whether the −117 mutation is a common polymorphism by use of oligonucleotide hybridization. We have synthesized one oligonucleotide specific for the mutant sequence and another 19mer specific for the normal sequence. We have then studied genomic DNA of various individuals. If the mutation and the phenotype are concordant, $^{A}\gamma\beta^{+}$ HPFH individuals, all heterozygous for an HPFH allele and a normal β-globin gene cluster, should carry both the mutant and the normal sequence in the region 117 nucleotides 5′ to the $^{A}\gamma$ gene, while individuals lacking the HPFH phenotype should have only the normal sequence. Indeed, our results demonstrate complete concordance of the HPFH phenotype with the −117 mutation.

Previously, some of us have shown concordance of the −202 mutation 5′ to the $^{G}\gamma$ gene with $^{G}\gamma\beta^{+}$ HPFH in blacks.[15] $^{A}\gamma\beta^{+}$ HPFH in Italians has been associated with a nucleotide substitution at position −196 5′ to the $^{A}\gamma$ gene.[10] That these mutations are so close to one another in sequence (−202 and −196) suggests that they may indeed be causative of the phenotype, and that a regulatory region exists around position −200 for the γ-globin genes. Complete concordance of the −117 mutation with $^{A}\gamma\beta^{+}$ HPFH increases the evidence that all of these mutations are causative of the HPFH phenotype.

However, even though there is complete concordance of the −117 mutation with $^{A}\gamma\beta^{+}$ HPFH, it is still remotely possible that the −117 change is merely a rare normal polymorphism and does not produce the HPFH phenotype. Indeed, in studies of restriction site and sequence polymorphisms in the β-globin gene cluster we have found one example of a rare normal polymorphism completely limited to chromosomes bearing a mutant allele. A β-thalassemia allele in Asian Indians has a silent mutation in exon 1 of the β-globin gene which produces a new Pst I site, while another mutation (a single nucleotide deletion 18 nucleotides downstream) actually causes β-thalassemia.[18] The Pst I change, though itself a normal variation, has not been found so far in a β^{A} allele. In the situation of the $^{A}\gamma\beta^{+}$ HPFH, however, the specific increase in $^{A}\gamma$ gene expression associated with a sequence change within the first 200 nucleotides 5′ to the $^{A}\gamma$ gene strongly suggests a cause-and-effect relationship. Definite proof that the −117 mutation causes HPFH may be obtained from studies in which this affected gene is inserted into transgenic mice or cultured cells.

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CONCISE REPORT

Thrombospondin in Essential Thrombocythemia

By Jack Lawler, Amos M. Cohen, Francis C. Chao, and Daniel J. Moriarty

Essential thrombocythemia is a myeloproliferative disorder characterized by frequent bleeding and thrombotic complications. On a molecular level, two abnormalities of platelet thrombospondin have been identified: abnormal glycosylation of the intact 185,000-dalton chain has been detected and a shortened form of the thrombospondin chain is present. We have used two monoclonal antibodies and Lens culinaris lectin to probe the structure of thrombospondin in the platelets from three patients with essential thrombocythemia; one patient with polycythemia vera and two patients with secondary thrombocytosis. The presence of abnormal thrombospondin fragments with molecular weights of 160,000 and 30,000 was detected in the intact platelets and in the supernatant from thrombin-treated platelets, in all of the individuals except one of the secondary thrombocytosis patients. Monoclonal antibody binding studies indicate that both fragments are produced by proteolysis at a single site, which results in the removal of a 30,000-dalton fragment from the NH_2-terminal. Lens culinaris lectin-binding studies revealed that some of the carbohydrate moieties of thrombospondin are near this cleavage site. The results are consistent with the hypothesis that the abnormal thrombospondin fragments observed under conditions of increased platelet production are due to increased susceptibility to proteolysis which, in turn, may be due to defective glycosylation.

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ESSENTIAL thrombocythemia is a myeloproliferative disorder characterized by frequent bleeding and thrombotic complications.[1-8] The platelets from these patients have been reported to be functionally abnormal in that they have decreased aggregation in response to thrombin, epinephrine, adenosine diphosphate, and collagen,[2-5,9,10] have reduced activity of the platelet lipoxygenase pathway,[6] are deficient in lipid peroxidation,[7] have reduced serotonin uptake,[2] and have decreased activation of platelet adenylate cyclase by prostaglandin D_2.[8] The platelets from these patients have some atypical morphology and can be enlarged.[2,5] On a molecular level, the platelets from essential thrombocythemia patients have a variable decrease in the content of membrane glycoprotein (GP) I and an increase in the content of GP IV and a 170,000-dalton polypeptide.[9-11] Clezardin et al[12] have reported that all of the platelet membrane glycoproteins are significantly less sialylated in essential thrombocythemia, as compared with controls. The greatest decrease in labeling of terminal sialic acid is observed for GPIb α and GPIIIa.[12] Booth et al[11] have shown that the 170,000-dalton polypeptide, which is increased in essential thrombocythemia, is related to thrombospondin. The electrophoretic pattern of nonreduced thrombospondin from these patients contained four closely spaced bands.[11] A similar pattern is observed when thrombospondin is exposed to mild proteolysis and probably represents intact thrombospondin and thrombospondin molecules in which one, two, or three chains have been proteolyzed.[13] Abnormal glycosylation of the intact 185,000-dalton chain of thrombospondin has also been detected using ^{125}I-labeled Lens culinaris lectin.[12] These data suggest that either the thrombospondin produced in these essential thrombocythemia patients is more labile to proteolysis or that there is an increase in protease activity in these platelets. Alternatively, a genetic mutation may give rise to the shortened form of the thrombospondin chain.

In the present study, two monoclonal anti-thrombospondin antibodies and Lens culinaris lectin are used to probe the structure of thrombospondin in essential thrombocythemia. An increase in the 30,000-dalton, NH_2-terminal, heparin-binding peptide has been detected in concert with the increase in a 160,000-dalton band. The data suggest that there is an increase in the susceptibility to proteolysis of a specific site in the thrombospondin molecule. Lens culinaris lectin binding studies indicate that there are carbohydrate moieties near this cleavage site, raising the possibility that defective glycosylation may lead to increased proteolysis.[12]

METHODS

Clinical material. This study was conducted according to the principles in the Declaration of Helsinki. The patients studied were from the Department of Hematology/Oncology of St Elizabeth's Hospital of Boston. Four patients with myeloproliferative disorders were studied. Three of these individuals (M.S., D.R., and A.H.) had essential thrombocythemia and one (R.R.) had polycythemia vera. The platelet counts of these patients varied from 0.5 to 1.5 × 10^9 platelets/mL during the course of this study. Over the same period, two patients (E.K. and M.N.) with secondary thrombocytosis were also studied. One of these patients (M.N.) had a splenectomy for trauma and the other (E.K.) had temporal arteritis.

Preparation of platelets. Venous blood from the patients and control normal volunteers was collected into sterile tubes containing the anticoagulant acid/citrate/dextrose (ACD, National Institutes of Health Formula A). Platelet-rich plasma was obtained by centrifuging whole blood at 500 × g for ten minutes. A one-fourth volume of ACD was added and the platelets were pelleted by centrifugation at 1,000 × g for 25 minutes. The platelets were washed once in 10 to 15 volumes of pH 6.5 buffer containing 0.102 mol/L NaCl, 3.9 mmol/L K_2HPO_4, 3.9 mmol/L Na_2HPO_4, 22 mmol/L NaH_2PO_4, and 5.5 mmol/L glucose. The platelets were resuspended in 15 mmol/L Tris-HCl (pH 7.6), 0.14 mol/L NaCl, and 5 mmol/L

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glucose at a cell count of 1 × 10^9 platelets/mL. The platelet suspension was treated with 1 mmol/L diisopropyl fluorophosphate (DFP) for ten minutes at 22 °C and was then solubilized with a one-fourth volume of electrophoresis sample buffer (pH 7.5) containing 0.25 mol/L Tris, 5% sodium dodecyl sulfate (SDS), 1.25 mol/L sucrose and 0.1 mol/L dithiothreitol at 100 °C for 60 seconds.

The supernatant from thrombin-treated platelets was prepared by exposing the platelet suspension, which had not received DFP treatment, to 0.25 U/mL of human thrombin (Sigma, St Louis) for two minutes at 22 °C. DFP (1 mmol/L, final concentration) was then added and the sample was spun at 1200 × g for five minutes. The supernatant was decanted and spun at 50,000 × g for 30 minutes.

Protein electrophoresis and blotting. SDS-Polyacrylamide gel electrophoresis (PAGE) was carried out on the discontinuous system of Laemmli[14] as described previously.[15] After SDS-PAGE, the proteins were electrophoretically transferred to nitrocellulose paper (BioRad, Richmond, Calif) at 50 volts for 24 to 72 hours in pH 8.3 buffer containing 25 mmol/L Tris, 192 mmol/L glycine, and 20% methanol. For monoclonal antibody binding studies, the nitrocellulose paper containing a replica of the gel was washed briefly in 0.15 mol/L NaCl and incubated with Tris-buffered saline (TBS-BSA) containing 15 mmol/L Tris-HCl (pH 7.6), 0.14 mol/L NaCl, and 5% bovine serum albumin for one hour at 22 °C. The protein blot was washed with five changes of 0.15 mol/L NaCl within a 30-minute interval and incubated with TBS-BSA containing monoclonal antithrombospondin antibodies (10 to 20 μL/10 mL TBS-BSA) for 16 hours at 22 °C. The production and epitope mapping of the monoclonal antibodies has been described previously.[15] The protein blots were washed again and incubated with goat anti-mouse IgG conjugated with horseradish peroxidase (10 μL/10 mL TBS-BSA: N.L. Cappel Laboratories, Cochranville, Pa) for one hour at 22 °C. After the final incubation, the protein blots were washed and placed in a substrate solution containing 40 mmol/L Tris-HCl (pH 7.6), 0.16 mol/L NaCl, 0.015% H_2O_2, 20% (vol/vol) methanol and 3.36 mmol/L 4-chloro-1-naphthol for one to five minutes for the detection of horseradish-peroxidase–conjugated anti-mouse IgG. For Lens culinaris lectin (Polysciences, Warrington, Pa) binding studies, the nitrocellulose paper containing a replica of the gel was washed briefly in 0.15 mol/L NaCl and incubated in 15 mmol/L Tris-HCl (pH 7.6), 0.14 mol/L NaCl, 2 mmol/L $CaCl_2$, and 3% periodic acid-treated BSA (TBS-P-BSA) for one hour at 22 °C.[16] The protein blots were washed as described above and incubated with TBS-P-BSA containing horseradish-peroxidase–conjugated Lens culinaris lectin (150 μL/30 mL TBS-P-BSA) for 16 hours at 22 °C. The blots were washed and incubated with the substrate solution described above.

RESULTS

The identification of abnormal thrombospondin fragments. The peptide compositions of the platelets from the essential thrombocythemia patient M.S. and a control normal volunteer are shown in Fig 1. At the protein loadings used, no differences are detectable by staining with Coomassie blue. We have electrophoretically transferred this pattern to nitrocellulose paper and have probed the replica with two murine monoclonal antithrombospondin antibodies, designated MA-I and MA-II. The epitope for MA-I is contained in a 47,000-dalton fragment of thrombospondin that is near the COOH-terminal of the 185,000-dalton thrombospondin chains.[15] The epitope for MA-II is contained in the NH_2-terminal, heparin-binding peptide.[15] This latter portion of the molecule is susceptible to proteolysis and is released as a 23,000- to 30,000-dalton fragment, depending on the enzyme used.[15,17–19]

When the platelet proteins from control normal volunteers or the essential thrombocythemia patients M.S., D.R., or A.H. were probed with MA-I or MA-II, the 185,000-dalton reduced chain of thrombospondin, was the principal band detected (Figs 1 and 2). When compared with the control samples, an increase in a 160,000-dalton band that binds MA-I was observed in these patients (Figs 1 and 2). The nearest band in the control samples had decreased electrophoretic mobility and staining intensity as compared with the 160,000-dalton band. When the blots were probed with MA-II, an increase in the 30,000-dalton band was observed in the platelets from the essential thrombocythemia patients, as compared with controls (Figs 1 and 2). Comparable levels of a 25,000-dalton band are observed for the controls and the essential thrombocythemia patients, with MA-II. The 160,000-, 30,000-, and 25,000-dalton fragments comigrate

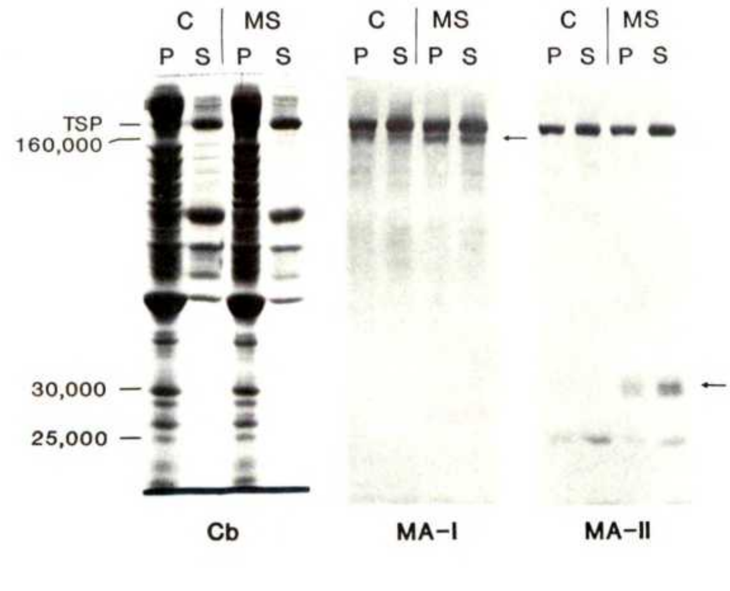

Fig 1. Peptide composition of the platelets from a control (C) normal volunteer and the essential thrombocythemia patient M.S. Solubilized platelets (P) and the supernatant (S) from thrombin-treated platelets were electrophoresed on 10% gels and stained with Coomassie blue (Cb) or electrophoretically transferred to nitrocellulose paper and probed with MA-I or MA-II. The electrophoretic mobility of the intact thrombospondin chain (TSP) and the 160,000-, 30,000-, and 25,000-dalton fragments are indicated on the left. The arrows indicate the position of the abnormal 160,000- and 30,000-dalton fragments detected by MA-I and MA-II, respectively.

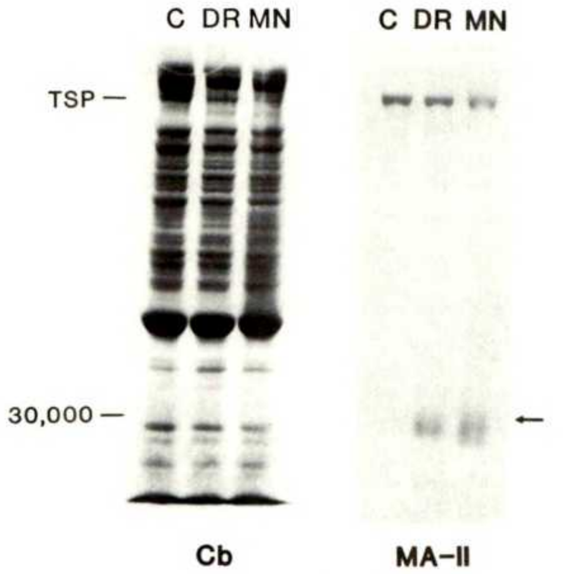

Fig 2. Peptide composition of platelets from a control (C) normal volunteer, the essential thrombocythemia patient D.R. and the secondary thrombocytosis patient M.N. The gels were stained with Coomassie blue (Cb) or electrophoretically transferred to nitrocellulose paper and probed with MA-II. The electrophoretic mobility of the intact thrombospondin chain (TSP) and the 30,000-dalton fragment are indicated on the left. The arrow indicates the position of the abnormal 30,000-dalton fragment detected by MA-II.

with fragments of thrombospondin produced by mild tryptic or chymotryptic digestion (data not shown). Similar results are obtained when the supernatants from thrombin-treated platelets are prepared from patients with essential thrombocythemia and control volunteers. That is, an increase in the 160,000-dalton fragment is detected with MA-I and an increase in the 30,000-dalton fragment is detected with MA-II in the essential thrombocythemia patients, relative to the controls (Fig 1).

To determine if the presence of the abnormal 160,000- and 30,000-dalton bands was specific to essential thrombocythemia, we also studied one patient (R.R.) with polycythemia vera and two patients (E.K. and M.N.) with secondary thrombocytosis. One of the secondary thrombocytosis patients (E.K.) was indistinguishable from control normal volunteers (data not shown). In contrast, patients R.R. and M.N. showed an increase in the 160,000-dalton and the 30,000-dalton bands detected by MA-I and MA-II, respectively (Fig 2).

Lens culinaris lectin binding to thrombospondin fragments. The results of limited chymotryptic digestion of thrombospondin at low enzyme-to-substrate ratios (1:10,000 and 1:1000) are shown in Fig 3. At an enzyme-to-substrate ratio of 1:10,000, a 30,000-dalton fragment is produced that contains the epitope for MA-II and a binding site for Lens culinaris lectin (Fig 3, lanes A). A 25,000-dalton fragment that contains the epitope for MA-II is also produced; however, this fragment does not bind Lens culinaris lectin. When the enzyme-to-substrate ratio is increased to 1:1000, a decrease in the 30,000-dalton fragment and a concomitant increase in the 25,000-dalton fragment is observed (Fig 3, lanes B). Despite the increase in the concentration of the 25,000-dalton fragment, no binding of Lens culinaris lectin could be detected under these conditions. At an enzyme-to-substrate ratio of 1:1000, a 70,000-dalton fragment is the principal Lens culinaris lectin-binding fragment.

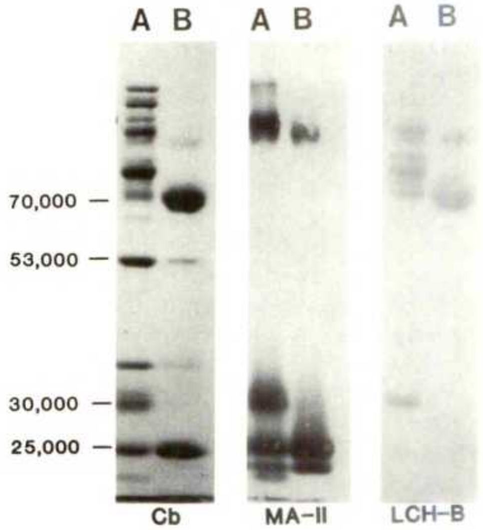

Fig 3. Chymotryptic digestion of thrombospondin. Thrombospondin was treated with 5 mmol/L EDTA and digested with chymotrypsin at enzyme-to-substrate ratios of 1:10,000 (A) or 1:1,000 (B) at 0 °C for 20 hours. The digests are electrophoresed on 10% gels and stained with Coomassie blue (Cb) or electrophoretically transferred to nitrocellulose and probed with MA-II or Lens culinaris lectin (LCH-B). The molecular weights of some of the principal fragments are indicated on the left.

DISCUSSION

The data presented here corroborates the data of Booth et al[11] in that there is an increase in a high molecular weight fragment of thrombospondin in the platelets from patients with essential thrombocythemia. The difference of 10,000 daltons between the reported molecular mass and the value of 160,000 daltons determined in this study is probably due to subtle differences in the calibration of the gel systems in the two laboratories. We have shown that the 160,000-dalton fragment includes the epitope for MA-I, but not the epitope for MA-II. In addition, we have detected the increase in a 30,000-dalton fragment that binds MA-II. Collectively, these data indicate that there is increased proteolysis of thrombospondin at a site that results in the release of a 30,000-dalton fragment from the NH_2-terminal of the thrombospondin chains.[15] This corresponds to site 1, which is between segments I and II described in our previous report.[15] The observation that both the 30,000- and 160,000-dalton fragments are present in the supernatant of thrombin-treated platelets suggest that both fragments are present in the α-granules. In the normal platelet, there seems to be proteolytic activity that results in the production of a 25,000-dalton fragment, which binds MA-II and is therefore at or near the NH_2-terminal of thrombospondin. The abnormal 160,000- and 30,000-dalton fragments were also detected in the polycythemia vera patient (R.R.) and one of the two patients (M.N.) with reactive thrombocytosis. Thus, these changes may generally occur under conditions of increased platelet production and are not specific to essential thrombocythemia.

Abnormal glycosylation of thrombospondin, in addition to other glycoproteins, has been reported in essential thrombocythemia.[9-12] We have used peroxidase-conjugated Lens culinaris lectin to locate the carbohydrate side chains that bind this lectin. In the chymotryptic digests, the 30,000-dalton fragment of the NH_2-terminal region binds Lens culinaris lectin, but the 25,000-dalton fragment does not. The 25,000-dalton fragment is probably derived from the 30,000-dalton fragment because both fragments contain the epitope for MA-II and an increase in the 25,000-dalton fragment occurs concomitant to the decrease in the 30,000-dalton fragment. The NH_2-terminal amino acid sequence of the 25,000-dalton fragment is identical to that of the intact thrombospondin chain.[18,19] Thus, the conversion from the 30,000-dalton fragment to the 25,000-dalton fragment probably occurs as a result of the proteolytic removal of 5,000 daltons from the COOH-terminal of the 30,000-dalton fragment. Since the 30,000-dalton fragment binds Lens culinaris lectin and the 25,000-dalton fragment does not, at least one carbohydrate moiety is probably located in the 5,000-dalton C-terminal portion of the 30,000-dalton fragment. A second binding site for Lens culinaris lectin is located in the 70,000-dalton fragment, which is derived from a portion of the molecule that is adjacent to the 30,000-dalton fragment.[19,20]

The data presented here indicates that there is increased susceptibility of thrombospondin to proteolysis in some of the myeloproliferative disorders and in secondary thrombocytosis. The site for proteolysis is in the immediate vicinity of a carbohydrate side chain. Abnormal glycosylation of thrombospondin in essential thrombocythemia has been reported.[12] Furthermore, carbohydrate moieties on proteins have been reported to confer resistance to proteolysis.[21] Thus, we propose that the increased susceptibility of the thrombospondin to proteolysis in primary and secondary thrombocytosis is a direct result of defective glycosylation.

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CONCISE REPORT

Retinoic Acid as Antileukemic Therapy in a Patient With Acute Promyelocytic Leukemia and *Aspergillus* Pneumonia

By S. Daenen, E. Vellenga, O.A. van Dobbenburgh, and M.R. Halie

A patient with acute promyelocytic leukemia (APL) and laboratory evidence of fibrinolysis who could not be treated with aggressive cytostatic regimens because of *Aspergillus* pneumonia was treated with *cis*-retinoic acid (RA), a substance that can induce differentiation and maturation of APL cells. After seven weeks of daily oral treatment, he went into complete remission, and signs of coagulopathy disappeared. Meanwhile, the *Aspergillus* pneumonia could be treated adequately. Based on the experience in this single patient, RA deserves further evaluation in the treatment of APL.

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RETINOIC ACID (RA) can inhibit the proliferation or induce the differentiation of leukemic cells in vitro[1,2] and in vivo,[3,4] but its place, if any, in the treatment of myeloid malignancies is not yet well defined. It seems that until now, RA is largely restricted to patients with acute promyelocytic leukemia (APL) who are resistant to cytostatic drugs[4] or with myelodysplastic syndromes for which no effective therapy is available at present.[5] We describe a patient with APL who developed pulmonary aspergillosis during the first (unsuccessful) remission-induction course and therefore could not be treated aggressively again. Use of 13-*cis*-RA (isotretinoin 80 mg daily) resulted in complete hematologic remission and permitted adequate treatment of the *Aspergillus* infection.

CASE HISTORY

A 33-year-old man was admitted to our department in December 1984 because of leukemia. He was well until three weeks previously, when a flu-like syndrome developed. On examination he appeared anemic and had purpuric spots on the lower extremities. Hemoglobin was 60 g/L, WBC 2.3 × 10^9/L with blast cells 14% and promyelocytes 28%, platelets 8 × 10^9/L. The bone marrow was hypercellular and comprised predominantly hypergranular promyelocytes (82%), many of them with Auer rods in fagots. A karyotype analysis failed to reveal chromosomal abnormalities, and in vitro culture of bone marrow gave no growth of hematopoietic stem cells. Coagulation studies revealed a low fibrinogen level (0.58 g/L; normal, 2–4 g/L), high fibrinogen degradation products (FDP, 80–120 μg/mL; normal, 0–10 μg/mL), and a high level of antithrombin III (150%; normal, 80–129%).

A diagnosis of APL complicated by a fibrinolytic syndrome was made. Antileukemic therapy consisted of Ara-C 100 mg/m^2 IV every 12 hours, 6-thioguanine 100 mg/m^2 orally every 12 hours for seven days, and adriamycin 40 mg/m^2 IV at day 5 to 7 (TAA). ε-Aminocaproic acid, platelet transfusions, and fresh frozen plasma were given as well. An episode of sinusitis was treated with systemic antibiotics. Two weeks after the start of chemotherapy, the body temperature became elevated and chest x-rays showed an infiltrate in the right lower lobe. This did not respond to antibiotics; on further examination (bronchial lavage and biopsy), *Aspergillus* pneumonia was diagnosed. However, serum antibodies for *Aspergillus* were negative. Antifungal treatment consisted of Amphotericin B in increasing dose to 2 × 20 mg IV and 5-fluorocytosine 10 gr orally per day, with gradual improvement of the general condition.

Five weeks after the start of cytostatic drugs, there was still cytopenia of the peripheral blood (Hb 98 g/L; WBC 0.9 × 10^9/L; platelets 38 × 10^9/L), and although the fibrinogen level had increased (5.22 g/L), FDP remained high (120–160 μg/mL). Repeat bone marrow examination demonstrated hypercellularity with a monotonous infiltration of promyelocytes as before, but with much fewer Auer rods (Fig 1A). Although we could not exclude the possibility of a delayed regeneration after cytostatic therapy or maturation arrest after trimethoprim-sulfamethoxazole, persistently active leukemia was considered more likely in the presence of marrow hypercellularity, absence of erythroid and megakaryocytoid elements, and persistent signs of coagulopathy. However, a new course with cytostatic drugs was considered too dangerous. 13-*cis*-RA (isotretinoin), a vitamin A analogue, was initiated in a daily oral dose of 80 mg. Four weeks later, the peripheral blood count was normal, apart from slight anemia (Hb 114 g/L; WBC 4.0 × 10^9/L; platelets 233 × 10^9/L), and the bone marrow had improved significantly, with Auer rods absent and myeloblasts and promyelocytes reduced to 6% and 16%, respectively. Levels of FDP were no longer elevated. Amphotericin B and 5-fluorocytosine were replaced by ketoconazole 2 × 200 mg daily, and isotretinoin was continued on an ambulatory base. Seven weeks after initiation of isotretinoin, the thoracic infiltrate had completely disappeared, Hb level had increased further (128 g/L), and the bone marrow was consistent with complete remission of APL (Fig 1B). Side effects of RA consisted of dry lips and small increases in SGPT level: 44 U/L (normal, 0–30 U/L).

The patient was seen regularly at the outpatient clinic while remaining on isotretinoin 80 mg daily and felt well throughout. At the last follow-up examination, seven months after the start of RA, his general condition was still excellent, and the peripheral blood was normal (Hb 149 g/L; WBC 4.5 × 10^9/L; platelets 185 × 10^9/L). However, the bone marrow contained 8% myeloblasts and a single promyelocyte with numerous Auer rods; the cellularity, the number of promyelocytes (6%), and the maturation of myeloid, erythroid, and megakaryocytoid cells, on the other hand, were normal. After a remission of nearly six months' duration, these were probably signs of an incipient relapse of the leukemic process.

DISCUSSION

Until less than ten years ago, *Aspergillus* infections in patients with acute leukemia were nearly always fatal,[6] and the occasional success with Amphotericin B, with[7] or without[8] granulocyte transfusions, was a subject for specific case reports. Currently, the prognosis is primarily determined by the outcome of the underlying disease[6] and the rapidity with which therapy is instituted.[9] When the leukemia goes into

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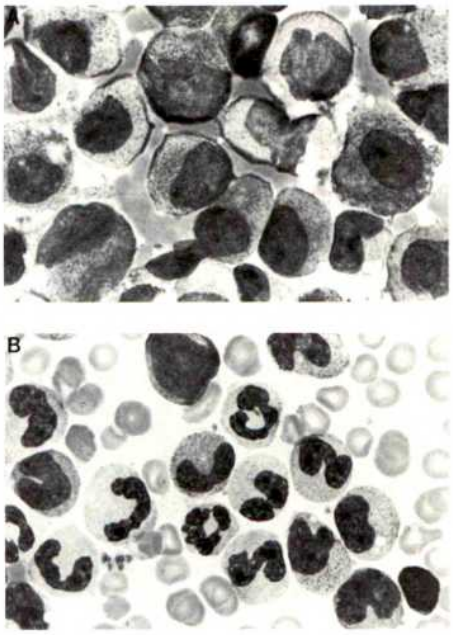

Fig 1. (A) Bone marrow after one TAA course. The predominance of promyelocytes (66%) and myeloblasts (10%) indicates that remission has not been achieved. (B) Bone marrow after seven weeks of RA. Myeloblast levels under 5%, promyelocyte levels of 7% to 8%, and normal differentiation to granulocytes are consistent with complete remission.

remission and Amphotericin B is started early, 17% to 64% of patients recover, depending on whether or not the infection is accompanied by the appearance of anti-*Aspergillus* antibodies in the serum.[10] However, even in apparently cured patients, the *Aspergillus* infection may recur many months later, upon recurrence of the leukemia.[7]

Obviously, the risks of aggressive chemotherapy in patients with active *Aspergillus* infection are considerable, and such patients are good candidates for alternative therapies. 13-*cis*-RA, an analogue of vitamin A, is one of the many agents that can induce differentiation and maturation in leukemic cells in vitro and one that can be used in vivo.[11] Other substances that are applicable in vivo are vitamin D metabolites, some cytostatic drugs in low dosage, purines and pyrimidines, and differentiation-inducing factors.[11] The differentiating effects of RA in vitro seem to be confined to the HL-60 cell line and to fresh leukemic cells from patients with APL.[1] However, Douer and Koeffler[2] also found an inhibition of clonal growth in vitro in 5 of 7 patients with acute myeloid leukemia without differentiation (AML); whether this applies to the in vivo situation is unknown. When the leukemic cells differentiate, they lose their suppressive action on normal bone marrow colony formation,[12] which might result in regrowth of residual normal bone marrow in vivo.

Flynn et al[3] were the first to demonstrate an effect of RA in a leukemic patient. However, this patient died of an *Aspergillus* infection two weeks later. Nilsson[4] recently reported on a patient with refractory APL who achieved a complete remission by a combination of RA and cytostatic drugs. Our patient achieved a complete remission on RA without cytostatic drugs. At the initiation of that therapy, we hoped only to slow the leukemic process and gain time to treat the *Aspergillus* infection before cytostatics had to be resumed. The response was much better than expected, and the patient went into complete remission, which lasted for nearly six months. Interestingly, signs of coagulopathy, probably caused by the release of fibrinolytic and proteolytic substances from the blasts,[13,14] disappeared quickly during RA therapy. This is a further argument that the leukemic cells are not killed under the influence of RA but that they differentiate and lose the capacity to release such fibrinolytic factors during maturation.

After seven months of therapy with RA, the bone marrow showed signs of incipient relapse. It is conceivable that a fraction of malignant cells not sensitive to the differentiating effect of RA has been selected by the therapy, because also in vitro only part of the leukemic cells seem to differentiate.[1] The leukemic cells at relapse also seemed to be less mature than the original promyelocytes.

Based on these preliminary observations in a single patient, we believe that the efficacy of RA therapy in APL deserves further evaluation.

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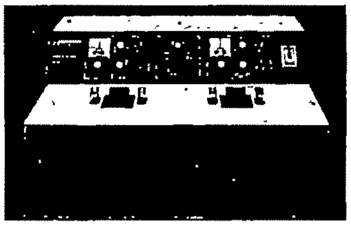

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(see page 1229 for program summary)

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WARNINGS: In the treatment of accidental overdosage of folic acid antagonists, leucovorin should be administered as promptly as possible. As the time interval between antifolate administration (e.g. methotrexate) and leucovorin rescue increases, leucovorin's effectiveness in counteracting hematologic toxicity diminishes.

PRECAUTIONS:

General: Following chemotherapy with folic acid antagonists, parenteral administration of leucovorin is preferable to oral dosing if there is a possibility that the patient may vomit and not absorb the leucovorin. In the presence of pernicious anemia a hematologic remission may occur while neurologic manifestations remain progressive. Leucovorin has no effect on other toxicities of methotrexate, such as the nephrotoxicity resulting from drug precipitation in the kidney.

Drug Interactions: Folic acid in large amounts may counteract the antiepileptic effect of phenobarbital, phenytoin and primidone, and increase the frequency of seizures in susceptible children.

Pregnancy: ***Teratogenic Effects:*** Pregnancy Category C. Animal reproduction studies have not been conducted with Wellcovorin. It is also not known whether Wellcovorin can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Wellcovorin should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Wellcovorin is administered to a nursing mother.

Pediatric Use: See "Drug Interactions".

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid.

OVERDOSAGE: Excessive amounts of leucovorin may nullify the chemotherapeutic effect of folic acid antagonists.

DOSAGE AND ADMINISTRATION: Leucovorin is a specific antidote for the hematopoietic toxicity of methotrexate and other strong inhibitors of the enzyme dihydrofolate reductase. Leucovorin rescue must begin within 24 hours of antifolate administration. A conventional leucovorin rescue dosage schedule is 10 mg/m^2 orally or parenterally followed by 10 mg/m^2 orally every six hours for seventy-two hours. If, however, at 24 hours following methotrexate administration the serum creatinine is 50% or greater than the pre-methotrexate serum creatinine, the leucovorin dose should be immediately increased to 100 mg/m^2 every three hours until the serum methotrexate level is below $5 \times 10^{-8}M$.[1,2]

The recommended dose of leucovorin to counteract hematologic toxicity from folic acid antagonists with less affinity for mammalian dihydrofolate reductase than methotrexate (i.e. trimethoprim, pyrimethamine) is substantially less and 5 to 15 mg of leucovorin per day has been recommended by some investigators.[3,4,5]

1. Bleyer WA: The Clinical Pharmacology of Methotrexate. *Cancer, 41*(1):36-51, 1978.
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5. Mahmoud AAF and Warren KS: Algorithms in the Diagnosis and Management of Exotic Disease. XX Toxoplasmosis. *J Infect Dis. 135*(3): 493-496, 1977.

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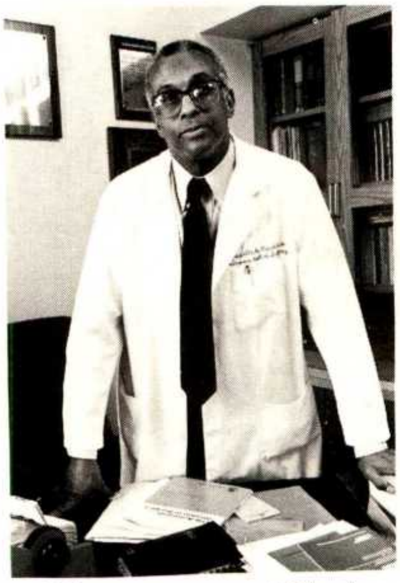

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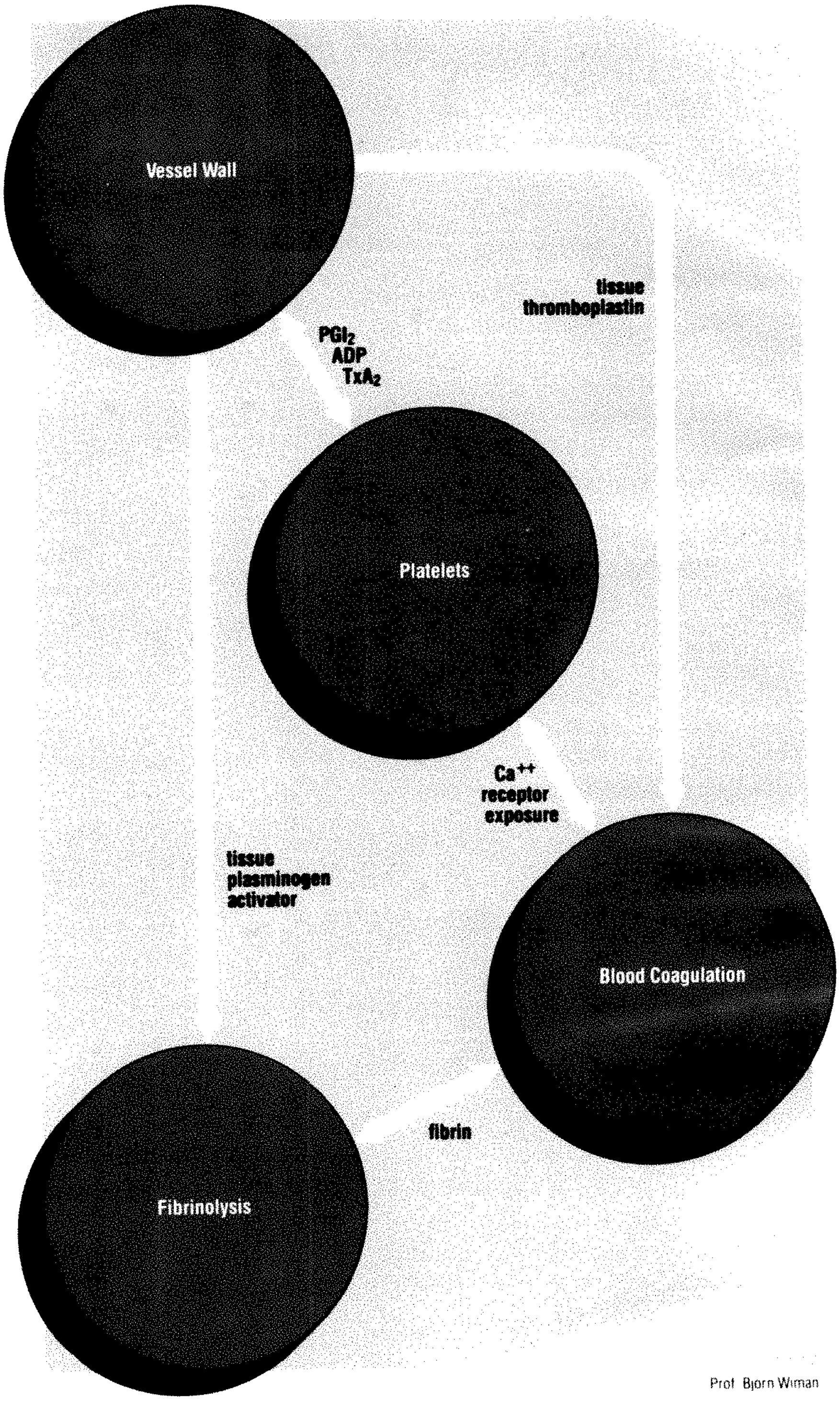

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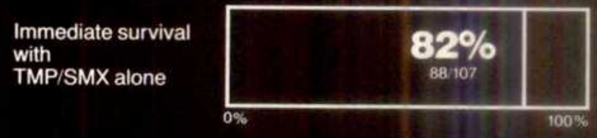

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Drug of choice in *PCP*[1]

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Before prescribing, please consult complete product information, a summary of which follows:

CONTRAINDICATIONS: Hypersensitivity to trimethoprim or sulfonamides; documented megaloblastic anemia due to folate deficiency; pregnancy at term and during the nursing period; infants less than two months of age.

WARNINGS: FATALITIES ASSOCIATED WITH THE ADMINISTRATION OF SULFONAMIDES, ALTHOUGH RARE, HAVE OCCURRED DUE TO SEVERE REACTIONS, INCLUDING STEVENS-JOHNSON SYNDROME, TOXIC EPIDERMAL NECROLYSIS, FULMINANT HEPATIC NECROSIS, AGRANULOCYTOSIS, APLASTIC ANEMIA AND OTHER BLOOD DYSCRASIAS.

BACTRIM SHOULD BE DISCONTINUED AT THE FIRST APPEARANCE OF SKIN RASH OR ANY SIGN OF ADVERSE REACTION. Clinical signs, such as rash, sore throat, fever, arthralgia, cough, shortness of breath, pallor, purpura or jaundice, may be early indications of serious reactions. In rare instances a skin rash may be followed by more severe reactions, such as Stevens-Johnson syndrome, toxic epidermal necrolysis, hepatic necrosis or serious blood disorder. Perform complete blood counts frequently.

BACTRIM SHOULD NOT BE USED IN THE TREATMENT OF STREPTOCOCCAL PHARYNGITIS. Clinical studies have documented that patients with group A β-hemolytic streptococcal tonsillopharyngitis have a greater incidence of bacteriologic failure when treated with Bactrim than with penicillin.

Bactrim I.V. Infusion contains sodium metabisulfite, which may cause allergic-type reactions in susceptible persons, especially asthmatics or atopic nonasthmatic persons.

PRECAUTIONS:

General: Give with caution to patients with impaired renal or hepatic function, possible folate deficiency (*e.g.*, elderly, chronic alcoholics, patients on anticonvulsants, with malabsorption syndrome, or in malnutrition states) and severe allergies or bronchial asthma. In glucose-6-phosphate dehydrogenase deficient individuals, hemolysis may occur, frequently dose-related.

Local irritation and inflammation due to extravascular infiltration of the infusion have been observed. If these occur, discontinue infusion and restart at another site.

Use in the Elderly: May be increased risk of severe adverse reactions in elderly, particularly with complicating conditions, *e.g.*, impaired kidney and/or liver function, concomitant use of other drugs. Severe skin reactions, generalized bone marrow suppression (see WARNINGS and ADVERSE REACTIONS) or a specific decrease in platelets (with or without purpura) are most frequently reported severe adverse reactions in elderly. In those concurrently receiving certain diuretics, primarily thiazides, increased incidence of thrombocytopenia with purpura reported. Make appropriate dosage adjustments for patients with impaired kidney function (see DOSAGE AND ADMINISTRATION).

Use in the Treatment of Pneumocystis Carinii Pneumonitis in Patients with Acquired Immunodeficiency Syndrome (AIDS): Because of unique immune dysfunction, AIDS patients may not tolerate or respond to Bactrim in same manner as non-AIDS patients. Incidence of side effects, particularly rash, fever, leukopenia, with Bactrim in AIDS patients treated for *Pneumocystis carinii* pneumonitis reported to be greatly increased compared with incidence normally associated with Bactrim in non-AIDS patients.

Laboratory Tests: Perform appropriate culture and susceptibility studies before and throughout treatment. Complete blood counts should be done frequently; if a significant reduction in the count of any formed blood element is noted, discontinue Bactrim. Perform urinalyses with careful microscopic examination and renal function tests during therapy, particularly for patients with impaired renal function.

Drug Interactions: In elderly patients concurrently receiving certain diuretics, primarily thiazides, an increased incidence of thrombocytopenia with purpura has been reported. Bactrim may prolong the prothrombin time in patients who are receiving the anticoagulant warfarin. Keep this in mind when Bactrim is given to patients already on anticoagulant therapy and reassess coagulation time. Bactrim may inhibit the hepatic metabolism of phenytoin. Given at a common clinical dosage, it increased the phenytoin half-life by 39% and decreased the phenytoin metabolic clearance rate by 27%. When giving these drugs concurrently, be alert for possible excessive phenytoin effect. Sulfonamides can displace methotrexate from plasma protein binding sites, thus increasing free methotrexate concentrations.

Drug/Laboratory Test Interactions: Bactrim, specifically the trimethoprim component, can interfere with a serum methotrexate assay as determined by the competitive binding protein technique (CBPA) when a bacterial dihydrofolate reductase is used as the binding protein. No interference occurs if methotrexate is measured by a radioimmunoassay (RIA). The presence of trimethoprim and sulfamethoxazole may also interfere with the Jaffé alkaline picrate reaction assay for creatinine, resulting in overestimations of about 10% in the range of normal values.

Carcinogenesis, Mutagenesis, Impairment of Fertility: Carcinogenesis: Long-term studies in animals to evaluate carcinogenic potential not conducted with Bactrim I.V. Infusion. *Mutagenesis:* Bacterial mutagenic studies not performed with sulfamethoxazole and trimethoprim in combination. Trimethoprim demonstrated to be nonmutagenic in the Ames assay. No chromosomal damage observed in human leukocytes cultured *in vitro* with sulfamethoxazole and trimethoprim alone or in combination; concentrations used exceeded blood levels of these compounds following therapy with Bactrim. Observations of leukocytes obtained from patients treated with Bactrim revealed no chromosomal abnormalities.

BACTRIM™ I.V. Infusion (trimethoprim and sulfamethoxazole/Roche)

Impairment of Fertility. Bactrim I.V. Infusion not studied in animals for evidence of impairment of fertility. Studies in rats at oral dosages as high as 70 mg/kg trimethoprim plus 350 mg/kg sulfamethoxazole daily showed no adverse effects on fertility or general reproductive performance.

Pregnancy: Teratogenic Effects: Pregnancy Category C. Trimethoprim and sulfamethoxazole may interfere with folic acid metabolism; use during pregnancy only if potential benefit justifies potential risk to fetus. Nonteratogenic Effects: See CONTRAINDICATIONS section.

Nursing Mothers: See CONTRAINDICATIONS section.

Pediatric Use. Not recommended for infants under two months (see CONTRAINDICATIONS section).

ADVERSE REACTIONS: Most common are gastrointestinal disturbances (nausea, vomiting, anorexia) and allergic skin reactions (such as rash and urticaria). **FATALITIES ASSOCIATED WITH THE ADMINISTRATION OF SULFONAMIDES, ALTHOUGH RARE, HAVE OCCURRED DUE TO SEVERE REACTIONS, INCLUDING STEVENS-JOHNSON SYNDROME, TOXIC EPIDERMAL NECROLYSIS, FULMINANT HEPATIC NECROSIS, AGRANULOCYTOSIS, APLASTIC ANEMIA AND OTHER BLOOD DYSCRASIAS (SEE WARNINGS SECTION).** Local reaction, pain and slight irritation on I.V. administration infrequent. Thrombophlebitis rarely observed.

Hematologic: Agranulocytosis, aplastic anemia, thrombocytopenia, leukopenia, neutropenia, hemolytic anemia, megaloblastic anemia, hypoprothrombinemia, methemoglobinemia, eosinophilia. *Allergic Reactions:* Stevens-Johnson syndrome, toxic epidermal necrolysis, anaphylaxis, allergic myocarditis, erythema multiforme, exfoliative dermatitis, angioedema, drug fever, chills, Henoch-Schoenlein purpura, serum sickness-like syndrome, generalized allergic reactions, generalized skin eruptions, conjunctival and scleral injection, photosensitivity, pruritus, urticaria and rash. Periarteritis nodosa and systemic lupus erythematosus have been reported. *Gastrointestinal:* Hepatitis (including cholestatic jaundice and hepatic necrosis), elevation of serum transaminase and bilirubin, pseudomembranous enterocolitis, pancreatitis, stomatitis, glossitis, nausea, emesis, abdominal pain, diarrhea, anorexia. *Genitourinary:* Renal failure, interstitial nephritis, BUN and serum creatinine elevation, toxic nephrosis with oliguria and anuria, crystalluria. *Neurologic:* Aseptic meningitis, convulsions, peripheral neuritis, ataxia, vertigo, tinnitus, headache. *Psychiatric:* Hallucinations, depression, apathy, nervousness. *Endocrine:* Sulfonamides bear certain chemical similarities to some goitrogens, diuretics (acetazolamide and the thiazides) and oral hypoglycemic agents; cross-sensitivity may exist. Diuresis and hypoglycemia have occurred rarely in patients receiving sulfonamides. *Musculoskeletal:* Arthralgia, myalgia. *Respiratory:* Pulmonary infiltrates. *Miscellaneous:* Weakness, fatigue, insomnia.

DOSAGE AND ADMINISTRATION: CONTRAINDICATED IN INFANTS LESS THAN TWO MONTHS OF AGE. CAUTION—BACTRIM I.V. INFUSION MUST BE DILUTED IN 5% DEXTROSE IN WATER SOLUTION PRIOR TO ADMINISTRATION. DO NOT MIX WITH OTHER DRUGS OR SOLUTIONS. RAPID INFUSION OR BOLUS INJECTION MUST BE AVOIDED.

DOSAGE *(Children and Adults):*

PNEUMOCYSTIS CARINII PNEUMONITIS: Total daily dose is 15 to 20 mg/kg (based on the trimethoprim component) given in three or four equally divided doses every 6 to 8 hours for up to 14 days. One investigator noted that a total daily dose of 10 to 15 mg/kg was sufficient in 10 adults with normal renal function.

SEVERE URINARY TRACT INFECTIONS AND SHIGELLOSIS: Total daily dose is 8 to 10 mg/kg (based on the trimethoprim component) given in two to four equally divided doses every 6, 8 or 12 hours for up to 14 days for severe urinary tract infections and 5 days for shigellosis. Maximum recommended daily dose is 60 ml. *Renal Impaired:* Creatinine clearance above 30 ml/min, give usual dosage; 15-30 ml/min, give one-half the usual regimen; below 15 ml/min, use not recommended.

Method of Preparation: Bactrim I.V. Infusion must be diluted. EACH 5 ML SHOULD BE ADDED TO 125 ML OF 5% DEXTROSE IN WATER. After diluting do not refrigerate; use within 6 hours. If a dilution of 5 ml/100 ml of 5% dextrose in water is desired, use within 4 hours. If there is cloudiness or evidence of crystallization, discard solution and prepare a fresh one.

Multidose Vials: After initial entry into the vial, use remaining contents within 48 hours. The following infusion systems are satisfactory: unit-dose glass containers; unit-dose polyvinyl chloride and polyolefin containers.

Dilution: ADD EACH 5 ML OF BACTRIM I.V. INFUSION TO 125 ML OF 5% DEXTROSE IN WATER.

NOTE: *When fluid restriction is desirable*, add each 5 ml to 75 ml of 5% dextrose in water; mix just prior to use and administer within two hours. If there is cloudiness or evidence of crystallization, discard solution and prepare a fresh one.

DO NOT MIX BACTRIM I.V. INFUSION—5% DEXTROSE IN WATER WITH DRUGS OR SOLUTIONS IN THE SAME CONTAINER.

ADMINISTRATION: Administer by I.V. infusion over 60 to 90 minutes. Avoid rapid infusion or bolus injection. Do not give intramuscularly.

HOW SUPPLIED: 5-ml ampuls, 5-ml vials and 10-ml vials—boxes of 10; 30-ml and 50-ml multidose vials—boxes of 1. Each 5 ml contains 80 mg trimethoprim (16 mg/ml) and 400 mg sulfamethoxazole (80 mg/ml) for infusion with 5% dextrose in water.

STORE AT ROOM TEMPERATURE (15°-30°C or 59°-86°F). DO NOT REFRIGERATE.

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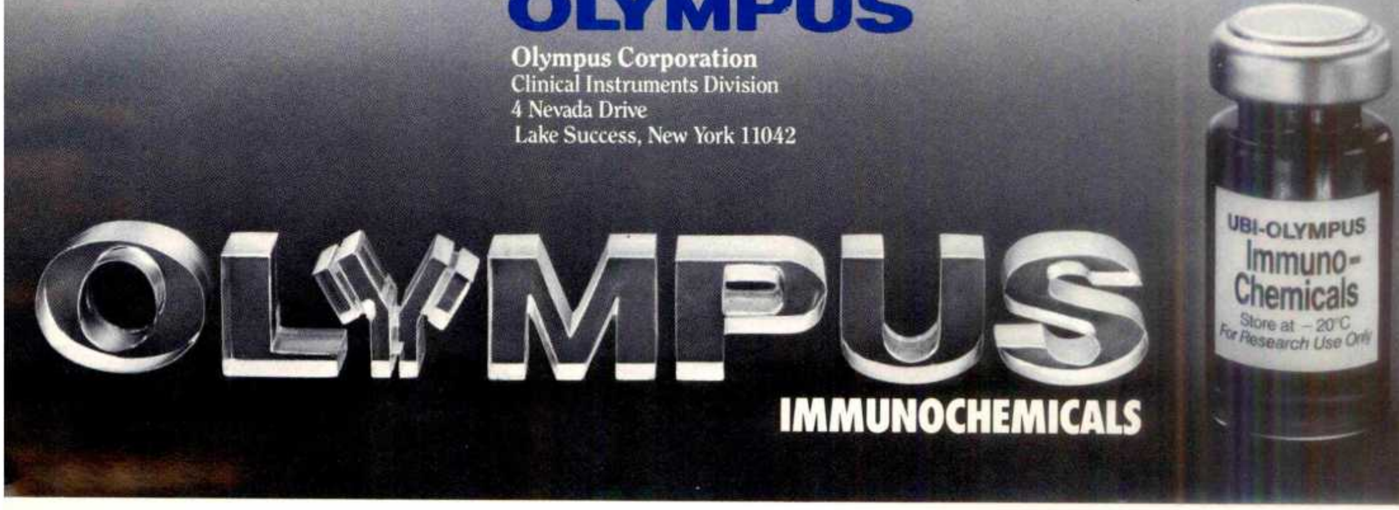

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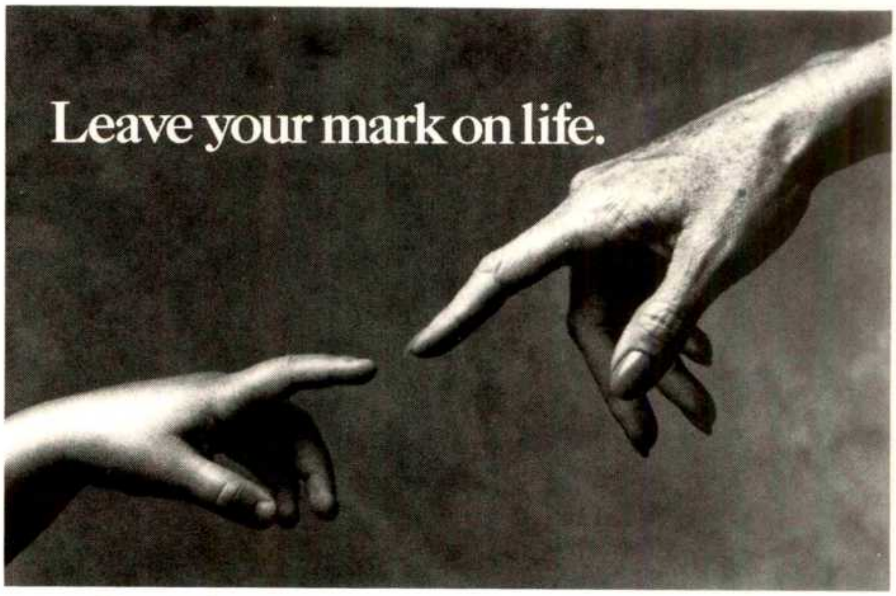
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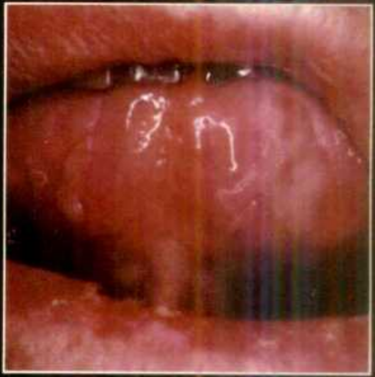

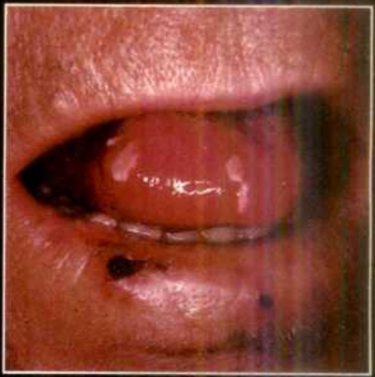

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INDICATIONS AND USAGE: Zovirax Sterile Powder is indicated for the treatment of initial and recurrent mucosal and cutaneous Herpes simplex (HSV-1 and HSV-2) infections in immunocompromised adults and children. It is also indicated for severe initial clinical episodes of herpes genitalis in patients who are not immunocompromised.

These indications are based on the results of several double-blind, placebo-controlled studies which evaluated the drug's effect on virus excretion, complete healing of lesions, and relief of pain.

Herpes Simplex Infections in Immunocompromised Patients

A multicenter trial of Zovirax Sterile Powder at a dose of 250 mg/M^2 every 8 hours (750 mg/M^2/day) for 7 days was conducted in 97 immunocompromised patients with oro-facial, esophageal, genital and other localized infections (50 treated with Zovirax and 47 with placebo). Zovirax significantly decreased virus excretion, reduced pain, and promoted scabbing and rapid healing of lesions.[1,2,3]

Initial Episodes of Herpes Genitalis

A controlled trial was conducted in 28 patients with severe initial episodes of herpes genitalis with a Zovirax dosage of 5 mg/kg every 8 hours for 5 days (12 patients treated with Zovirax and 16 with placebo). Significant treatment effects were seen in elimination of virus from lesions and in reduction of healing times.[4]

In a similar study, 15 patients with initial episodes of genital herpes were treated with Zovirax 5 mg/kg every 8 hours for 5 days and 15 with placebo. Zovirax decreased the duration of viral excretion, new lesion formation, duration of vesicles and promoted more rapid healing of all lesions.[5]

Diagnosis

The use of appropriate laboratory diagnostic procedures will help to establish the etiologic diagnosis. Positive cultures for Herpes simplex virus offer a reliable means for confirmation of the diagnosis. In initial episodes of genital herpes, appropriate examinations should be performed to rule out other sexually transmitted diseases. Whereas cutaneous lesions associated with Herpes simplex infections are often characteristic, the finding of multinucleated giant cells in smears prepared from lesion exudate or scrapings may assist in the diagnosis.[6]

CONTRAINDICATIONS: Zovirax Sterile Powder is contraindicated for patients who develop hypersensitivity to the drug.

WARNINGS: Zovirax Sterile Powder is intended for intravenous infusion only, and should not be administered topically, intramuscularly, orally, subcutaneously or in the eye. Intravenous infusions must be given over a period of at least 1 (one) hour to prevent renal tubular damage (see PRECAUTIONS and DOSAGE AND ADMINISTRATION).

PRECAUTIONS:

General: The recommended dosage, frequency and length of treatment should not be exceeded (See DOSAGE AND ADMINISTRATION).

Although the aqueous solubility of acyclovir sodium (for infusion) is >100 mg/ml, precipitation of acyclovir crystals in renal tubules can occur if the maximum solubility of free acyclovir (2.5 mg/ml at 37°C in water) is exceeded or if the drug is administered by bolus injection. This complication causes a rise in serum creatinine and blood urea nitrogen (BUN), and a decrease in renal creatinine clearance. Ensuing renal tubular damage can produce acute renal failure.

Abnormal renal function (decreased creatinine clearance) can occur as a result of acyclovir administration and depends on the state of the patient's hydration, other treatments, and the rate of drug administration. Bolus administration of the drug leads to a 10% incidence of renal dysfunction, while in controlled studies, infusion of 5 mg/kg (250 mg/M^2) over an hour was associated with a lower frequency — 4.6%. Concomitant use of other nephrotoxic drugs, pre-existing renal disease, and dehydration make further renal impairment with acyclovir more likely. In most instances, alterations of renal function were transient and resolved spontaneously or with improvement of water and electrolyte balance, drug dosage adjustment or discontinuation of drug administration. However, in some instances, these changes may progress to acute renal failure.

Administration of Zovirax by intravenous infusion must be accompanied by adequate hydration. Since maximum urine concentration occurs within the first 2 hours following infusion, particular attention should be given to establishing sufficient urine flow during that period in order to prevent precipitation in renal tubules.

When dosage adjustments are required they should be based on estimated creatinine clearance (See DOSAGE AND ADMINISTRATION).

Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes characterized by either lethargy, obtundation, tremors, confusion, hallucinations, agitation, seizures or coma. Zovirax should be used with caution in those patients who have underlying neurologic abnormalities and those with serious renal, hepatic, or electrolyte abnormalities or significant hypoxia. It should also be used with caution in patients who have manifested prior neurologic reactions to cytotoxic drugs or those receiving concomitant intrathecal methotrexate or interferon.

Exposure of HSV isolates to acyclovir *in vitro* can lead to the emergence of less sensitive viruses. These viruses usually are deficient in thymidine kinase (required for acyclovir activation) and are less pathogenic in animals. Similar isolates have been observed in 6 severely immunocompromised patients during the course of controlled and uncontrolled studies of intravenously administered Zovirax. These occurred in patients with congenital severe combined immunodeficiencies or following bone marrow transplantation. The presence of these viruses was not associated with a worsening of clinical illness and, in some instances, the virus disappeared spontaneously. The possibility of the appearance of less sensitive viruses must be borne in mind when treating such patients. The relationship between the *in vitro* sensitivity of herpesviruses to acyclovir and clinical response to therapy has yet to be established.

Drug Interactions: Co-administration of probenecid with acyclovir has been shown to increase the mean half-life and the area under the concentration-time curve. Urinary excretion and renal clearance were correspondingly reduced. Clinical experience has identified no other significant interactions resulting from administration of other drugs concomitantly with Zovirax Sterile Powder.

Carcinogenesis, Mutagenesis, Impairment of Fertility: Acyclovir was tested in lifetime bioassays in rats and mice at single daily doses of 50, 150 and 450 mg/kg given by gavage. There was no statistically significant difference in the incidence of tumors between treated and control animals, nor did acyclovir appear to shorten the latency of tumors. In 2 *in vitro* cell transformation assays, used to provide preliminary assessment of potential oncogenicity in advance of these more definitive lifetime bioassays in rodents, conflicting results were obtained. Acyclovir was positive at the highest dose used in one system and the resulting morphologically transformed cells formed tumors when inoculated into immunosuppressed, syngeneic, weanling mice. Acyclovir was negative in another transformation system.

No chromosome damage was observed at maximum tolerated parenteral doses of 100 mg/kg acyclovir in rats or Chinese hamsters; higher doses of 500 and 1000 mg/kg were clastogenic in Chinese hamsters. In addition, no activity was found in a dominant lethal study in mice. In 9 of 11 microbial and mammalian cell assays, no evidence of mutagenicity was observed. In 2 mammalian cell assays (human lymphocytes and L5178Y mouse lymphoma cells *in vitro*), positive responses for mutagenicity and chromosomal damage occurred, but only at concentrations at least 25 times the acyclovir plasma levels achieved in man.

Acyclovir does not impair fertility or reproduction in mice at oral doses up to 450 mg/kg/day. In female rabbits treated subcutaneously with acyclovir subsequent to mating, there was a statistically significant decrease in implantation efficiency but no concomitant decrease in litter size at a dose of 50 mg/kg/day.

Pregnancy: Teratogenic Effects. Pregnancy Category C. Acyclovir was not teratogenic in the mouse (450 mg/kg/day, p.o.), rabbit (50 mg/kg/day, s.c.) or rat (50 mg/kg/day, s.c.).

Although maximum tolerated doses were tested in teratology studies, the plasma levels obtained did not exaggerate maximum plasma levels that might occur with clinical use of intravenous acyclovir.

There have been no adequate and well-controlled studies in pregnant women. Acyclovir should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Zovirax (acyclovir sodium) sterile powder is administered to a nursing woman.

ADVERSE REACTIONS: The most frequent adverse reactions reported during controlled clinical trials of Zovirax in 64 patients were inflammation or phlebitis at the injection site following infiltration of the I.V. fluid in 9 (14.0%), transient elevations of serum creatinine in 3 (4.7%), and rash or hives in 3 (4.7%). Less frequent adverse reactions were diaphoresis, hematuria, hypotension, headache and nausea, each of which occurred in 1 patient (1.6%). Of the 63 patients receiving placebo, 3 (4.8%) experienced inflammation/phlebitis and 3 (4.8%) experienced rash or itching. Hematuria and nausea were experienced by placebo recipients at the same frequency.

Among 51 immunocompromised patients, one, a bone marrow transplant recipient with pneumonitis, developed seizures, cerebral edema, coma and expired with changes consistent with cerebral anoxia on postmortem biopsy; another immunocompromised patient exhibited coarse tremor and clonus.

Additional adverse reactions were reported in uncontrolled trials. The most frequent adverse reaction was elevated serum creatinine. This occurred in 9.8 percent of patients, usually following rapid (less than 10 minutes) intravenous infusion. Less frequent adverse experiences were thrombocytosis and jitters, each in 0.4% of patients.

Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes characterized by either lethargy, obtundation, tremors, confusion, hallucinations, agitation, seizures or coma (see PRECAUTIONS).

OVERDOSAGE: Overdosage has been reported following administration of bolus injections, or inappropriately high doses, and in patients whose fluid and electrolyte balance was not properly monitored. This has resulted in elevations in BUN, serum creatinine and subsequent renal failure.

Precipitation of acyclovir in renal tubules may occur when the solubility (2.5 mg/ml) in the intratubular fluid is exceeded (see PRECAUTIONS). A six hour hemodialysis results in a 60% decrease in plasma acyclovir concentration. Data concerning peritoneal dialysis are incomplete but indicate that this method may be significantly less efficient in removing acyclovir from the blood. In the event of acute renal failure and anuria, the patient may benefit from hemodialysis until renal function is restored (see DOSAGE AND ADMINISTRATION).

DOSAGE AND ADMINISTRATION: CAUTION — RAPID OR BOLUS INTRAVENOUS AND INTRAMUSCULAR OR SUBCUTANEOUS INJECTION MUST BE AVOIDED.

Dosage: ***MUCOSAL AND CUTANEOUS HERPES SIMPLEX (HSV-1 and HSV-2) INFECTIONS IN IMMUNOCOMPROMISED PATIENTS*** — 5 mg/kg infused at a constant rate over 1 hour, every 8 hours (15 mg/kg/day) for 7 days in adult patients with normal renal function. In children under 12 years of age, more accurate dosing can be attained by infusing 250 mg/M^2 at a constant rate over 1 hour, every 8 hours (750 mg/M^2/day) for 7 days.

SEVERE INITIAL CLINICAL EPISODES OF HERPES GENITALIS — The same dose given above — administered for 5 days.

Therapy should be initiated as early as possible following onset of signs and symptoms.

PATIENTS WITH ACUTE OR CHRONIC RENAL IMPAIRMENT: Refer to DOSAGE AND ADMINISTRATION section for recommended doses, and adjust the dosing interval as indicated in the table below.

Creatinine Clearance (ml/min/1.73M^2)	Dose (mg/kg)	Dosing Interval (hours)
>50	5	8
25-50	5	12
10-25	5	24
0-10	2.5	24

Hemodialysis: For patients who require dialysis, the mean plasma half-life of acyclovir during hemodialysis is approximately 5 hours. This results in a 60% decrease in plasma concentrations following a 6 hour dialysis period. Therefore, the patient's dosing schedule should be adjusted so that a dose is administered after each dialysis.

Method of Preparation: Each 10 ml vial contains acyclovir sodium equivalent to 500 mg of acyclovir. The contents of the vial should be dissolved in 10 ml of sterile water for injection or bacteriostatic water for injection containing benzyl alcohol yielding a final concentration of 50 mg/ml of acyclovir (pH approximately 11). Shake the vial well to assure complete dissolution before measuring and transferring each individual dose. DO NOT USE BACTERIOSTATIC WATER FOR INJECTION CONTAINING PARABENS. It is incompatible with Zovirax Sterile Powder and may cause precipitation.

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EXAMPLES OF REFERENCES

Journal article, one or more authors:

1. Golomb HM, Vardiman J, Sweet DL, Simon D, Variakojis D: Hairy cell leukemia: Evidence for the existence of a spectrum of functional capabilities. Br J Haematol 38:161, 1978

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8. Seeler RA: Sickle cell anemia monthly variations. Blood 47:879, 1976 (letter)

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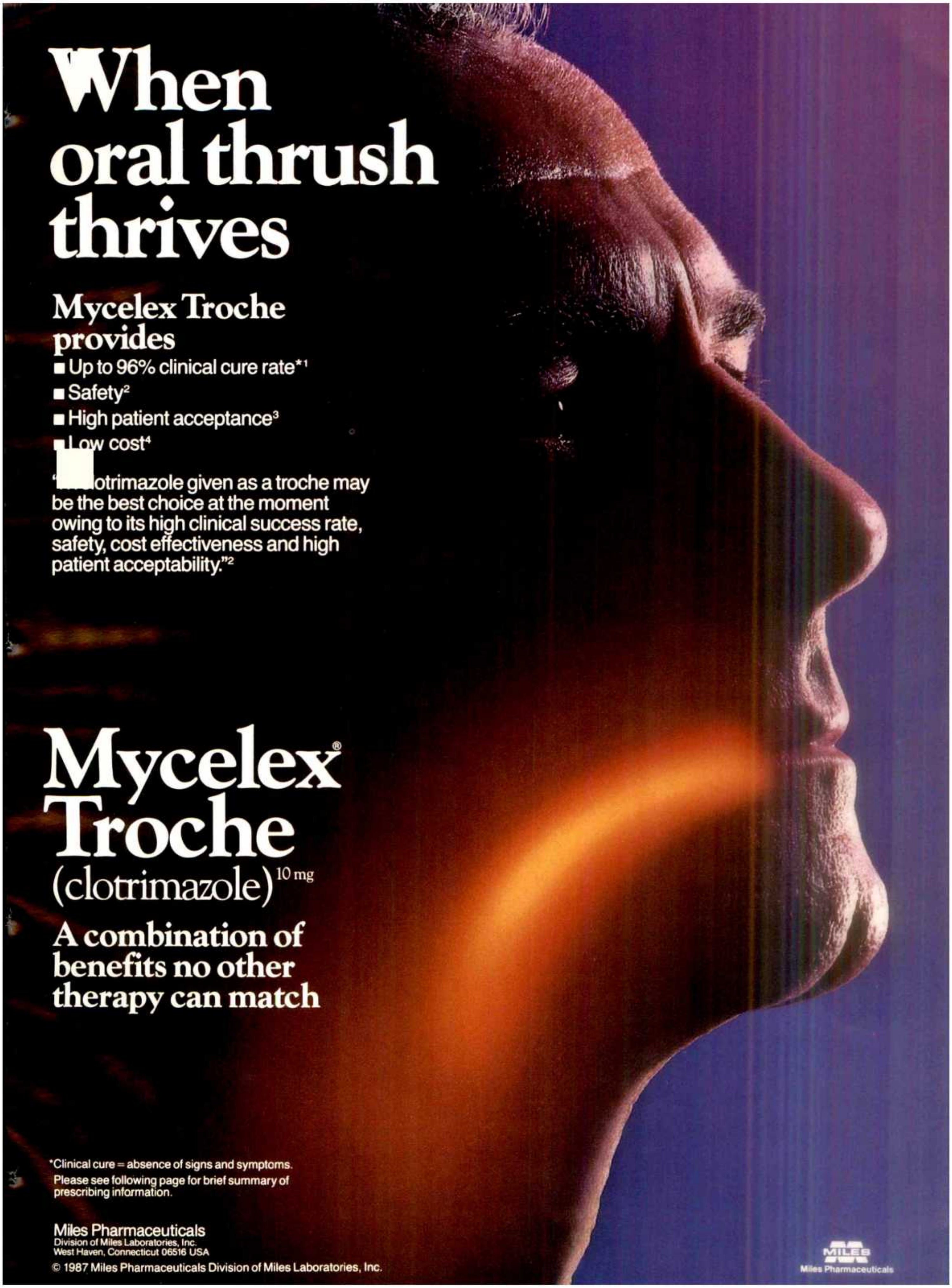

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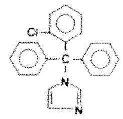
Each Mycelex® Troche contains 10 mg clotrimazole [1-(o-chloro-α, α-diphenylbenzyl) imidazole], a synthetic antifungal agent, for topical use in the mouth.
Structural Formula:



Chemical Formula:
$C_{22}H_{17}ClN_2$

The troche dosage form is a large, slowly-dissolving tablet (lozenge) containing 10 mg of clotrimazole dispersed in dextrose, microcrystalline cellulose, povidone, and magnesium stearate.

Clinical Pharmacology
Clotrimazole is a broad-spectrum antifungal agent that inhibits the growth of pathogenic yeasts by altering the permeability of cell membranes. It exhibits fungicidal activity *in vitro* against *Candida albicans* and other species of the genus *Candida*. No single-step or multiple-step resistance to clotrimazole has developed during successive passages of *Candida albicans* in the laboratory.
After oral administration of a 10 mg clotrimazole troche to healthy volunteers concentrations sufficient to inhibit most species of *Candida* persist in saliva for up to three hours following the approximately 30 minutes needed for a troche to dissolve. The long term effective concentration of drug in saliva appears to be related to the slow release of clotrimazole from the oral mucosa to which the drug is apparently bound. Repetitive dosing at three hour intervals maintains effective salivary levels above the minimum inhibitory concentration of most strains of *Candida*. In another study the mean serum concentrations were 4.98 ± 3.7 and 3.23 ± 1.4 nanograms/ml at 30 and 60 minutes respectively.

Indications and Usage
Mycelex® Troches are indicated for the local treatment of oropharyngeal candidiasis. The diagnosis should be confirmed by a KOH smear and/or culture prior to treatment.

Contraindications
Mycelex® Troches are contraindicated in patients who are hypersensitive to any of its components.

Warning
Mycelex® Troches are not indicated for the treatment of systemic mycoses.

Precautions
Abnormal liver function tests have been reported in patients treated with clotrimazole troches; elevated SGOT levels were reported in about 15% of patients in the clinical trials. In most cases the elevations were minimal and it was often impossible to distinguish effects of clotrimazole from those of other therapy and the underlying disease (malignancy in most cases). Periodic assessment of hepatic function is advisable particularly in patients with pre-existing hepatic impairment.
Since patients must be instructed to allow each troche to dissolve slowly in the mouth in order to achieve maximum effect of the medication, they must be of such an age and physical and/or mental condition to comprehend such instructions.
Carcinogenesis: An 18 month dosing study with clotrimazole in rats has not revealed any carcinogenic effect.
Usage in Pregnancy: Pregnancy Category C. Clotrimazole has been shown to be embryotoxic in rats and mice when given in doses 100 times the adult human dose (in mg/kg), possibly secondary to maternal toxicity. The drug was not teratogenic in mice, rabbits, and rats when given in doses up to 200, 180, and 100 times the human dose.
Clotrimazole given orally to mice from nine weeks before mating through weaning at a dose 120 times the human dose was associated with impairment of mating, decreased number of viable young, and decreased survival to weaning. No effects were observed at 60 times the human dose. When the drug was given to rats during a similar time period at 50 times the human dose, there was a slight decrease in the number of pups per litter and decreased pup viability.
There are no adequate and well controlled studies in pregnant women. Clotrimazole troches should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Pediatric Use
Safety and effectiveness of clotrimazole in children below the age of 3 years have not been established; therefore, its use in such patients is not recommended.

Adverse Reactions
Abnormal liver function tests have been reported in patients treated with clotrimazole troches; elevated SGOT levels were reported in about 15% of patients in the clinical trials (see Precautions section).
Nausea and vomiting was reported in about one in twenty patients.

Overdosage
No data available.

Drug Abuse and Dependence
No data available.

Dosage and Administration
Mycelex® Troches are administered only as a lozenge that must be slowly dissolved in the mouth. The recommended dose is one troche five times a day for fourteen consecutive days. Only limited data are available on the safety and effectiveness of the clotrimazole troche after prolonged administration; therefore, therapy should be limited to short term use, if possible.

How Supplied
Mycelex® Troches, white discoid, uncoated tablets are supplied in bottles of 70 and 140. Mycelex Troches are also available for institutional use in foil packages of 70 tablets. Each tablet will be identified with the following: Miles 095

Store below 86°F (30°C), avoid freezing

References: 1. Yap B-S, Bodey GP: Oropharyngeal candidiasis treated with a troche form of clotrimazole. *Arch Intern Med* 139:656-657, 1979. **2.** Quintiliani R, Owens NJ, Quercia RA, *et al*: Treatment and prevention of oropharyngeal candidiasis. *Am J Med* 77(4D):44-48, 1984. **3.** Data on file, Miles Pharmaceuticals. **4.** *Drug Topics Red Book, Annual Pharmacists' Reference*, Medical Economics Co., Oradell, NJ, 1987, pp. 397, 398, 411

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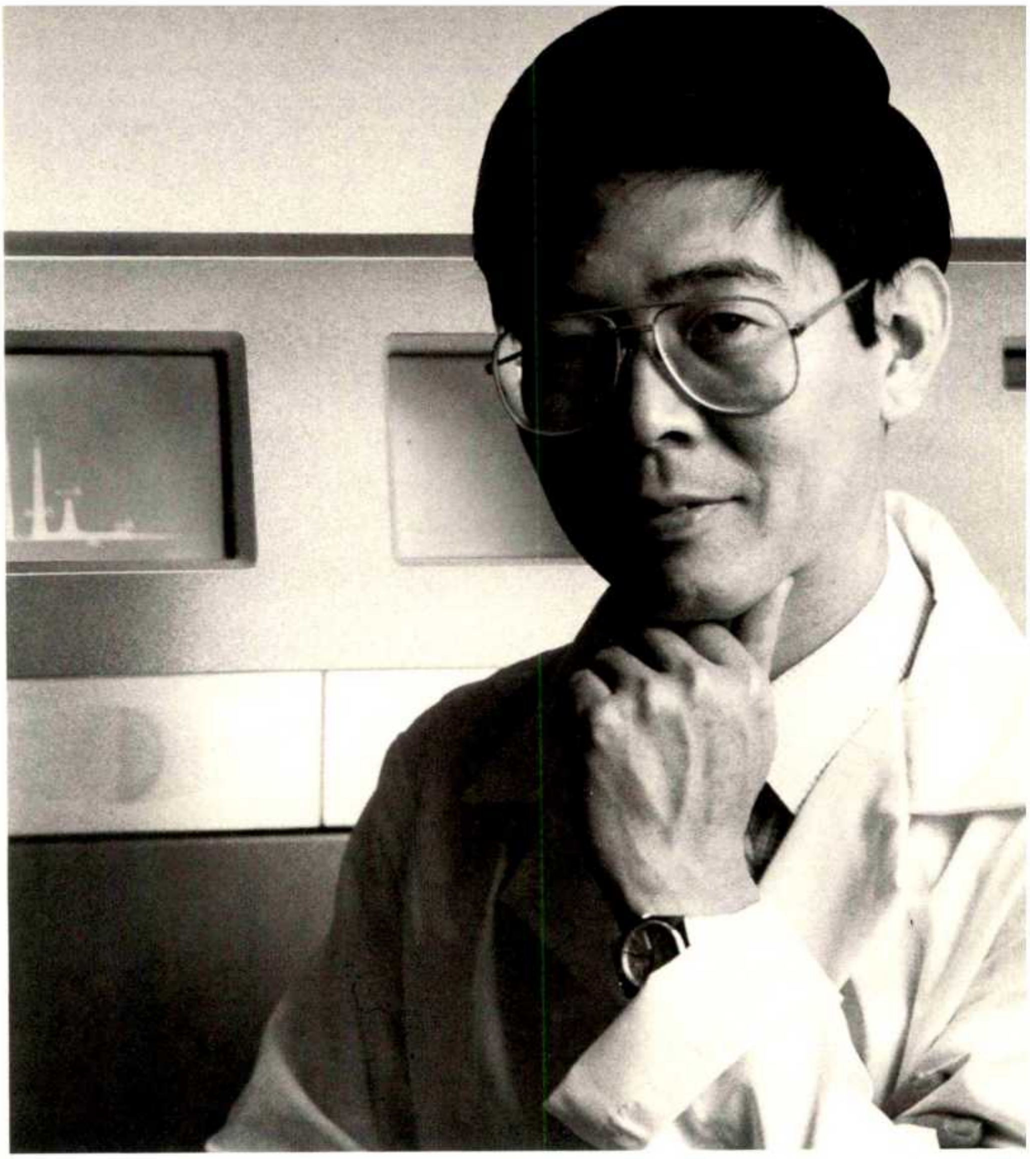
malignant cells."*



Peter Keng, Ph.D.
Associate Professor of Radiation Oncology
University of Rochester
School of Medicine and Dentistry

Dr. Keng was interviewed and photographed June 23, 1987, at his laboratory in the Cancer Center at Rochester, New York.

There's no substitute for experienc

Dr. Peter Keng of the University of Rochester School of Medicine and Dentistry, New York, heads the Cancer Center flow cytometry laboratory where his primary research consists of DNA analysis of tumors. In this recent interview, Dr. Keng discusses the advantages of the EPICS® Profile clinical flow cytometry analyzer from his experience in terms of the particular needs and applications of his laboratory.

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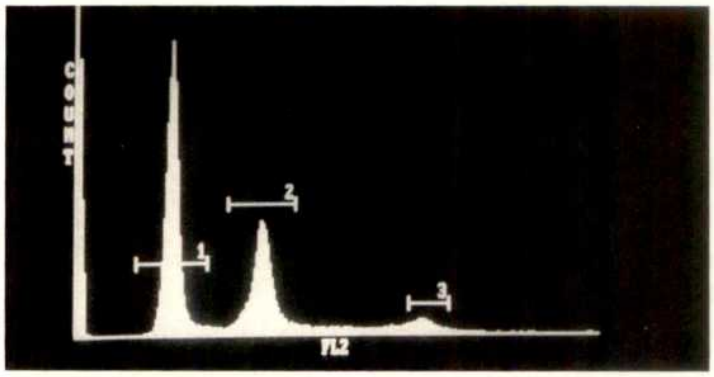

Propidium iodide stained nuclei from a dissociated human hepatoma tumor.
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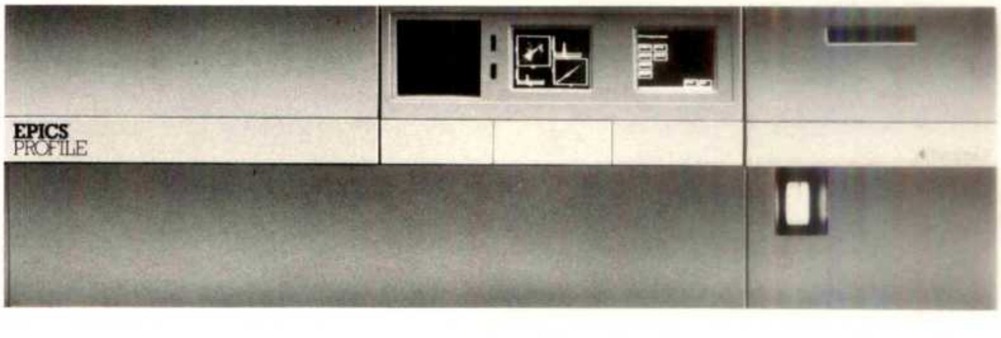

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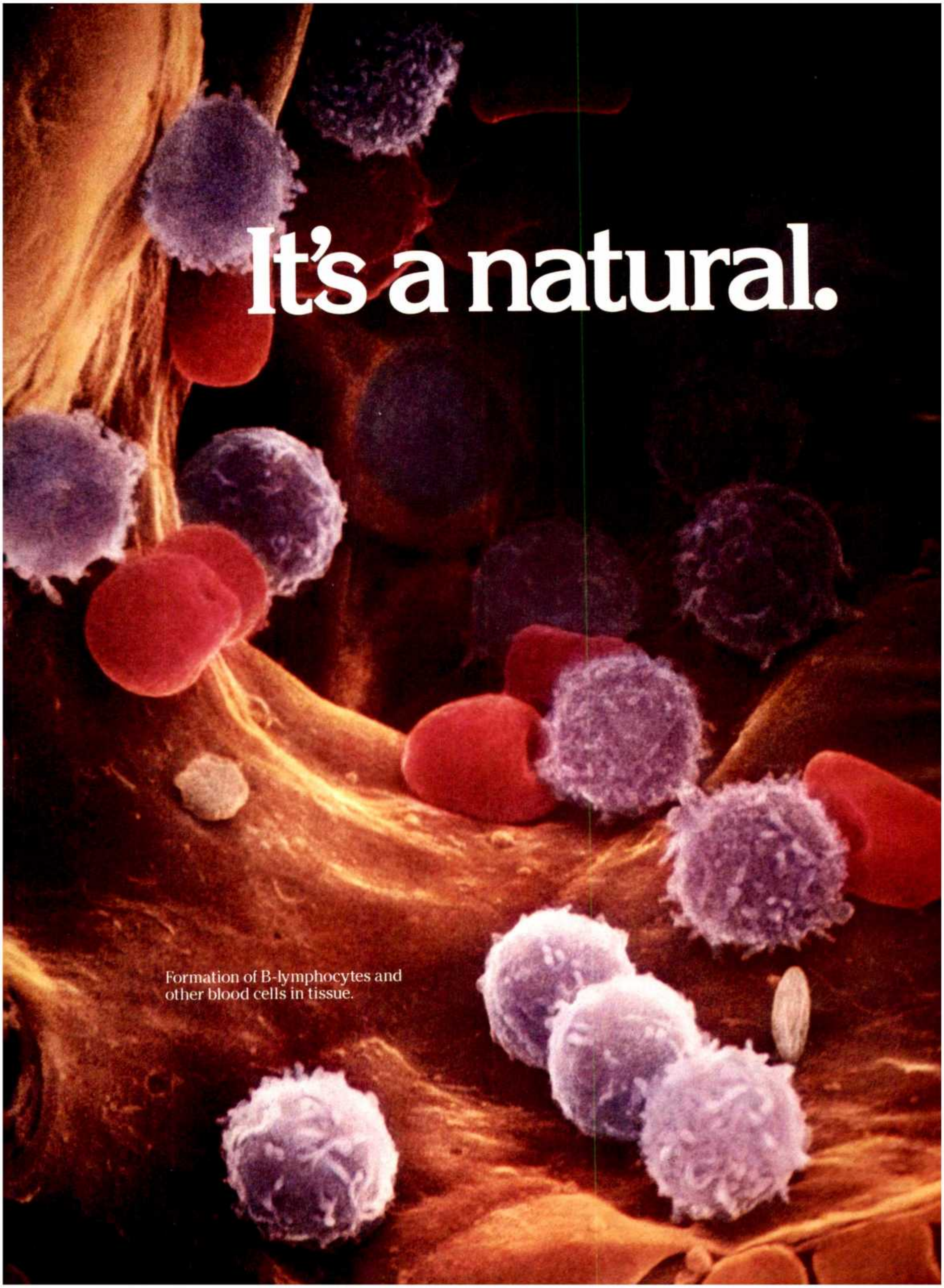


Formation of B-lymphocytes and other blood cells in tissue.

For ITP Patients

GAMIMUNE® N
Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25

For patients with acute and chronic idiopathic thrombocytopenic purpura (ITP), maintaining normal platelet levels may be a life-threatening problem. They often need fast, aggressive therapy that will elevate platelet counts rapidly. They need Gamimune® N.

IGIV treatment with Gamimune® N may effect a therapeutic increase in platelets in both children and adults with ITP and, in some cases, allows the possibility of major surgery.[1] In one study,[1] 62.5%(10 of 16) adult patients and 83.3% (5 of 6) pediatric patients demonstrated a substantial response to an initial course of therapy with Gamimune® N.*†

Gamimune® N contains at least 98% gamma globulin and is the only ready-to-use liquid IGIV available. Cutter's patented fractionation method (based on Cohn-Oncley methods 6 and 9) assures Gamimune® N contains no heterologous proteins, with no detectable levels of protein aggregates or vasoactive enzymes.[2] And studies show *in vitro* inactivation of HIV (HTLV-III virus‡ during production of Gamimune® N.

Gamimune® N. Enhancing the natural process with fast, aggressive therapy for ITP in both children and adults.

---

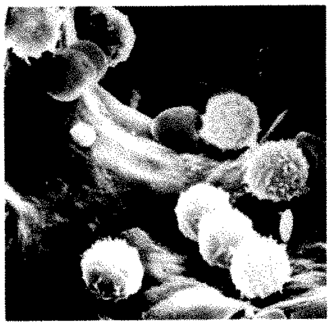
*It is not possible to predict which patients with ITP will respond to therapy.

†In patients who respond to therapy, a rise in platelet count is generally rapid (within one to five days), transient (most often lasting from several days to several weeks), and should not be considered curative.

‡The Cohn-Oncley process used to produce Gamimune® N has been challenged *in vitro* with a virus spike of 1 x 10^5 TCID (Tissue Culture Infectious Doses) of HIV (HTLV-III) and found to remove and/or inactivate the viral challenge. In addition, Gamimune® N undergoes an incubation step as a final container which has been shown to effect an additional 1 x 10^3 to 1 x 10^4 reduction TCID (Tissue Culture Infectious Doses) of HIV (HTLV-III).[3]

1. Gamimune® N Product License Application
2. Lundblad JL, Mitra G, Sternberg MM, et al. Comparative studies of impurities in intravenous immunoglobulin preparations. *Rev Infect Dis* 1986;8(suppl 4): S382-S390.
3. Mitra G, Wong MF, Mozen M, et al. Elimination of infectious retroviruses during preparation of immunoglobulins. *Transfusion* 1986;26:394-397

See adjacent page for brief summary of prescribing information.



GAMIMUNE® N

Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25

CLINICAL PHARMACOLOGY

Gamimune® N supplies a broad spectrum of opsonic and neutralizing IgG antibodies for the prevention or attenuation of a wide variety of infectious diseases. As Gamimune® N is administered intravenously, essentially 100% of the infused IgG antibodies are immediately available in the recipient's circulation. A relatively rapid fall in serum IgG level in the first week post-infusion is to be expected; this decrease averages 50% of the peak level achieved immediately post-infusion and is mainly due to the equilibration of IgG between the plasma and the extravascular space. The in vivo half-life of Gamimune® N equals or exceeds the three week half-life reported for IgG in the literature, but individual patient variation in half-life has been observed. Thus, this variable as well as the amount of immune globulin administered per dose is important in determining the frequency of administration of the drug for each individual patient.

The intravenous administration of solutions of maltose has been studied by several investigators. Healthy subjects tolerated the infusions well, and no adverse effects were observed at a rate of 0.25 g maltose/kg body weight per hour. In safety studies conducted by Cutter Biological, infusions of 10% maltose administered at 0.27-0.62 g maltose/kg per hour to normal subjects produced either mild side effects (e.g., headache) or no adverse reaction. Following intravenous administrations of maltose, maltose was detected in the peripheral blood; there was a dose-dependent excretion of maltose and glucose in the urine and a mild diuretic effect. These alterations were well tolerated without significant adverse effects. The highest recommended infusion rate, 0.08 mL/kg body weight per minute (see DOSAGE AND ADMINISTRATION), is equivalent to 0.48 g maltose/kg body weight per hour.

The buffer capacity of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N is 16.5 mEq/L (~ 0.3 mEq/g protein); a dose of 150-400 mg/kg (3-8 mL/kg) body weight therefore represents an acid load of 0.0495-0.1320 mEq/kg body weight. The total buffering capacity of whole blood in a normal individual is 45-50 mEq/L of blood, or 3.6 mEq/kg body weight. Thus, the acid load delivered in the largest dose of Gamimune® N would be neutralized by the buffering capacity of whole blood alone, even if the dose were infused instantaneously. (An infusion usually lasts several hours.)

In Phase I human studies, no change in arterial blood pH measurements was detected following the intravenous administration of Gamimune® N at a dose of 150 mg/kg body weight; following a dose of 400 mg/kg body weight in 37 patients, there were no clinically important differences in mean venous pH or bicarbonate measurements in patients who received Gamimune® N compared with those who received a chemically modified intravenous immunoglobulin preparation with a pH of 6.8.

In patients with limited or compromised acid-base compensatory mechanisms, consideration should be given to the effect of the additional acid load Gamimune® N might present.

INDICATIONS AND USAGE

Immunodeficiency Syndromes: Gamimune® N is indicated for the maintenance treatment of patients who are unable to produce sufficient amounts of IgG antibodies. Usage of Gamimune® N may be preferred to that of intramuscular immunoglobulin preparations, especially in patients who require an immediate increase in intravascular immunoglobulin levels, in patients with a small muscle mass, and in patients with bleeding tendencies in whom intramuscular injections are contraindicated. It may be used in disease states such as congenital agammaglobulinemia (e.g., X-linked agammaglobulinemia), common variable hypogammaglobulinemia, X-linked immunodeficiency with hyper IgM and in severe combined immunodeficiency.

Idiopathic Thrombocytopenic Purpura (ITP): Investigations in both children and adults have shown that Gamimune® N may initiate a therapeutic increase in the platelet count. In clinical studies of Gamimune® N, five of six (83.3%) children and 10 of 16 (62.5%) adults with acute or chronic ITP demonstrated clinically significant increments in the platelet count during or following an initial treatment course with Gamimune® N at a dose of 400 mg/kg body weight daily for five days. The duration of the platelet rise following treatment of ITP with Gamimune® N was variable, ranging from several days up to 12 months or more. Several ITP patients demonstrated continuing responsiveness over many months to intermittent Gamimune® N 400 mg/kg body weight single dose maintenance courses. Two of three children with acute ITP treated with Gamimune® N rapidly went into complete remission. However, childhood ITP may respond spontaneously without treatment. Four patients with refractory ITP were able to undergo major surgical procedures as a result of the rapid rise in platelet count associated with Gamimune® N treatment.

In addition, one patient with severe thrombocytopenia due to post-transfusion purpura (PL^{A1} alloimmune antibody with platelet anti PL^{A1} specificity) also responded to treatment with Gamimune® N, at a dose of 400 mg/kg body weight daily for 5 days; there was a rapid rise in the platelet count commencing on the third day of treatment.

It is presently not possible to predict which patients with ITP will respond to therapy, although the increase in platelet counts in children seems to be better than that of adults. In clinical situations in which a rapid rise in platelet count is needed to control bleeding or to allow a patient with ITP to undergo surgery, administration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N should be considered; in patients in whom a response is achieved, the rise of platelets is generally rapid (within 1–5 days), transient (most often lasting from several days to several weeks) and should not be considered curative. In some patients who relapse, a maintenance dose of Gamimune® N administered every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels (see DOSAGE AND ADMINISTRATION).

CONTRAINDICATIONS

Gamimune® N is contraindicated in individuals who are known to have had an anaphylactic or severe systemic response to Immune Globulin (Human). Individuals with selective IgA deficiencies who have known antibody against IgA (anti-IgA antibody) should not receive Gamimune® N since these patients may experience severe reactions to the IgA which may be present.

WARNINGS

Gamimune® N should be administered only intravenously as the intramuscular and subcutaneous routes have not been evaluated.

Gamimune® N may, on rare occasions, cause a precipitous fall in blood pressure and a clinical picture of anaphylaxis, even when the patient is not known to be sensitive to immune globulin preparations. These reactions may be related to the rate of infusion. Accordingly, the infusion rate given under DOSAGE AND ADMINISTRATION should be closely followed, at least until the physician has had sufficient experience with a given patient. The patient's vital signs should be monitored continuously and careful observation made for any symptoms throughout the entire infusion. Epinephrine should be available for the treatment of an acute anaphylactic reaction.

PRECAUTIONS

General: Any vial that has been entered should be used promptly. Partially used vials should be discarded. Do not use if turbid. Solution which has been frozen should not be used.

Drug Interactions: If dilution is required, Gamimune® N may be diluted with 5% dextrose in water (D5/W). No other drug interactions or compatibilities have been evaluated. It is recommended that infusion of Gamimune® N be given by a separate line, by itself, without mixing with other intravenous fluids or medications the patient might be receiving.

Pregnancy Category C: Animal reproduction studies have not been conducted with Gamimune® N. It is not known whether Gamimune® N can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Gamimune® N should be given to a pregnant woman only if clearly needed.

ADVERSE REACTIONS

In a study of 37 patients with immunodeficiency syndromes receiving Gamimune® N at a monthly dose of 400 mg/kg body weight, reactions were seen in 5.2% of the infusions of Gamimune® N. Symptoms reported with Gamimune® N included malaise, a feeling of faintness, fever, chills, headache, nausea, vomiting, chest tightness, dyspnea and chest, back or hip pain. In addition, mild erythema following infiltration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N at the infusion site was reported in some cases.

In further studies of Gamimune® N in the treatment of both adult and pediatric patients with ITP, systemic reactions were noted in only 4 of 154 (2.6%) infusions, and all but one occurred at rates of infusion greater than 0.04 mL/kg body weight per minute. The symptoms reported included chest tightness, a sense of tachycardia (pulse was 84 beats per minute), and a burning sensation in the head; these symptoms were all mild and transient. Erythema, pain, phlebitis, or eczematous reactions at the infusion site were also reported following infusion of Gamimune® N to adult ITP patients. These reactions occurred in 11.6% of the infusions of Gamimune® N and in 43.8% of the adult ITP population. No pediatric patients experienced localized reactions at the infusion site.

In the studies undertaken to date, other types of reactions have not been reported with Gamimune® N. It may be, however, that adverse effects will be similar to those previously reported with intravenous and intramuscular immunoglobulin administration. Potential reactions, therefore, may also include anxiety, flushing, wheezing, abdominal cramps, myalgias, arthralgia, and dizziness; rash has been reported only rarely. Reactions to intravenous immunoglobulin tend to be related to the rate of infusion.

True anaphylactic reactions to Gamimune® N may occur in recipients with documented prior histories of severe allergic reactions to intramuscular immunoglobulin, but some patients may tolerate cautiously administered intravenous immunoglobulin without adverse effects. Very rarely an anaphylactoid reaction may occur in patients with no prior history of severe allergic reactions to either intramuscular or intravenous immunoglobulin.

DOSAGE AND ADMINISTRATION

Immunodeficiency Syndromes: The usual dosage of Gamimune® N for prophylaxis in immunodeficiency syndromes is 100–200 mg/kg (2–4 mL/kg) of body weight administered approximately once a month by intravenous infusion. If the clinical response is inadequate, or the level of IgG achieved in the circulation is felt to be insufficient, the dosage may be given more frequently or increased as high as 400 mg/kg (8 mL/kg) body weight.

Idiopathic Thrombocytopenic Purpura: An increase in platelet count has been observed in children and some adults with acute or chronic ITP receiving Gamimune® N, 400 mg/kg body weight daily for five days. A response usually occurs within several days and is maintained for a variable period of time. In general a response is seen less often in adults than in children. In patients who have shown a response, a maintenance dose of Gamimune® N of 400 mg/kg body weight administered as a single dose every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels.

Investigations indicate that Gamimune® N is well-tolerated and less likely to produce side effects when infused at the indicated rate. It is recommended that Gamimune® N be infused by itself at a rate of 0.01 to 0.02 mL/kg body weight per minute for 30 minutes; if well tolerated, the rate may be **gradually** increased to a maximum of 0.08 mL/kg body weight per minute. If side effects occur, the rate may be reduced, or the infusion interrupted until symptoms subside. The infusion may then be resumed at the rate which is comfortable for the patient.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

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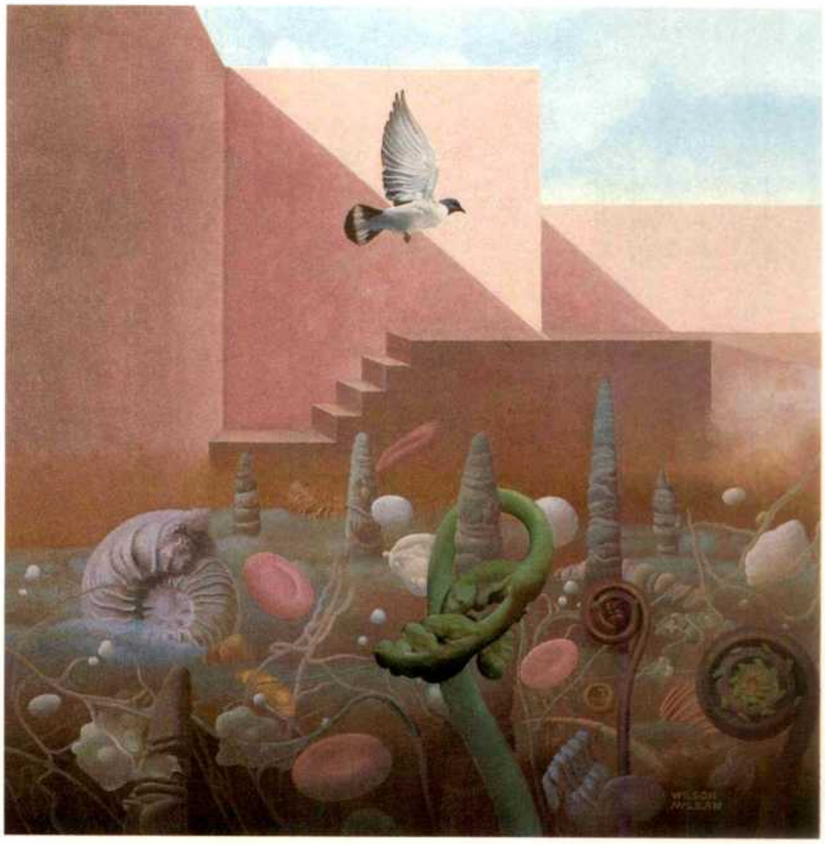

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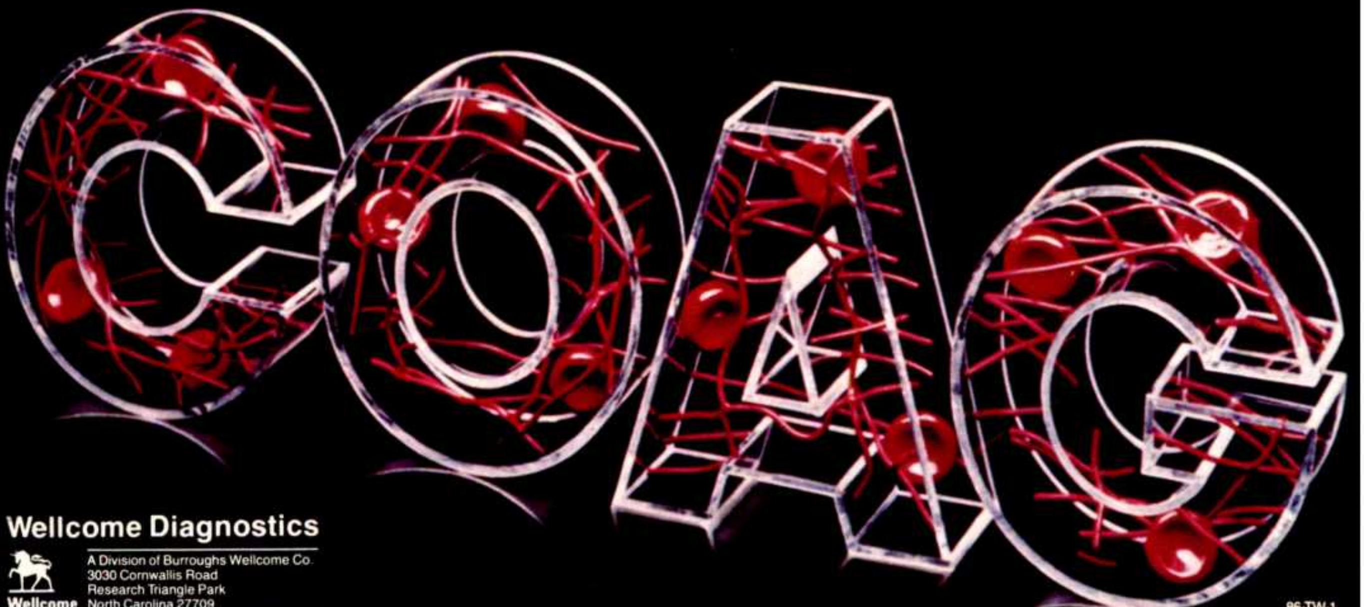

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REVIEW

von Willebrand Factor and von Willebrand Disease

By Zaverio M. Ruggeri and Theodore S. Zimmerman

Progress has occurred in the past several years in the understanding of the structure and function of von Willebrand factor (vWF). This multimeric glycoprotein exhibits a dual role, that of mediating platelet adhesion and aggregation onto thrombogenic surfaces, and that of functioning as carrier in plasma for the factor VIII procoagulant protein. New insights into the nature of the several functional domains of vWF have led to the identification of the regions of the molecule that interact with factor VIII, heparin, the glycoprotein Ib of platelets, and collagen. Alterations of vWF are the cause of von Willebrand disease (vWD), a congenital bleeding disorder. In the majority of patients, the plasma levels of vWF are decreased, but there is no demonstrable structural or functional alteration of the protein. In other patients, however, the structure of vWF is abnormal. This review summarizes the current knowledge on vWF and vWD.

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IN 1926, Erik von Willebrand, a German professor, described the congenital bleeding disorder that bears his name after studying several members of a family from Föglö, an island of the Åland archipelago in the Gulf of Bothnia.[1] After 45 years, in 1971, the protein known today as von Willebrand factor (vWF) was first detected immunologically and named, at the time, factor VIII-related antigen.[2] Although this relatedness remains a valid concept, since factor VIII and vWF form a noncovalent complex in blood, the two proteins are now known to be distinct molecules. Today, vWF has been cloned,[3-6] its primary amino acid sequence has been determined,[7] and its complex molecular structure and multiple functions are being understood in detail. This review summarizes the current knowledge on vWF and von Willebrand disease (vWD).

VON WILLEBRAND FACTOR

Biosynthesis of vWF

Endothelial cells and megakaryocytes are the site of synthesis of vWF.[8,9] The gene encoding for this protein is located on chromosome 12.[3,6] The corresponding mRNA is 8.5 to 9 kilobases (kb) in length and encodes for a protein constituted of 2,813 amino acid residues.[10] This protein represents pre-pro-vWF. The initial translation product rapidly dimerizes in the endoplasmic reticulum by interchain disulfide bonding, presumably involving the carboxyl termini of two subunits,[11] and undergoes a complex series of processing steps, including removal of a signal peptide 22 amino acids long[10] and glycosylation.[12] Moreover, a large polypeptide, 741 amino acid residues long, is cleaved from the amino terminus of the pro-subunit,[13] an event that may occur after dimerization. The cleaved pro-polypeptide is secreted as a distinct protein[13,14] or is found in the α-granules of platelets[15] and corresponds to the previously described von Willebrand antigen II.[16] Its function as a plasma and platelet protein remains unknown. After this cleavage, the mature vWF subunit is constituted of 2,050 amino acid residues. Its primary amino acid sequence has also been determined by classical Edman degradation.[7]

vWF dimers are assembled into multimers of larger size by formation of interchain disulfide bonds, presumably involving the amino termini of adjacent subunits in a head-to-head configuration.[11] This process may require a cellular environment with acidic pH and may normally occur after cleavage of the propolypeptide sequence corresponding to von Willebrand antigen II.[12,17-19] Persistence of this uncleaved sequence in a recently described variant form of von Willebrand disease (vWD), however, has been found not to interfere with multimerization.[20] The mature vWF protein of endothelial cells, assembled into an array of multimeric forms, is either secreted constitutively[21] or stored in Weibel-Palade bodies for release induced by appropriate stimuli.[22] Secretion of vWF by the vascular endothelium can be either into the circulation or abluminal,[23,24] in which case the protein becomes part of the subendothelial matrix. The vWF of megakaryocytes, on the other hand, is packaged into the α-granules of platelets[25] for secretion at the time of platelet activation.[25,26] It is not known when and how vWF complexes to factor VIII.

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Structure–Function Relationships of vWF

vWF participates in hemostasis by mediating the adhesion of platelets to exposed subendothelium and promoting the formation of platelet thrombi at sites of vascular injury.[27,28] Moreover, vWF forms a noncovalent complex with factor VIII[29,30] and prevents rapid removal of this procoagulant protein from the circulation.[31] Thus, vWF interacts with cell receptors, insoluble components of the subendothelium, and a circulating protein. This multiplicity of binding functions justifies its definition as an "adhesive" protein.

Two distinct receptors on the platelet membrane bind vWF, the glycoprotein Ib and the heterodimer complex formed by the glycoproteins IIb and IIIa.[32] The glycoprotein Ib-binding domain of vWF resides in a 52/48-kiloDalton (kD) tryptic fragment extending from residues Val_{449} to Lys_{728}.[33] This same fragment also contains binding domains for heparin[34] and collagen.[35] The glycoprotein IIb/IIIa complex-binding domain is believed to be represented by the tetrapeptide Arg-Gly-Asp-Ser (residues 1,744 through 1,747),[36] a sequence common to several other adhesive proteins, including fibrinogen[37] and fibronectin.[38] A second heparin binding domain exists in the amino terminal region of the constituent subunit, within residues 1 through 300,[39] and a second collagen binding domain exists in the middle region of the subunit, within residues 911 through 1,114.[40,41] The amino terminal region of the vWF subunit also contains the site to which the factor VIII procoagulant protein binds.[42] A tentative representation of the binding domains of the vWF subunit, based on the experimental evidence obtained to date, is shown in Fig 1.

Formation of the initial monolayer of platelets covering injured endothelial surfaces, particularly in segments of the vasculature where high shear wall stress prevails, is believed to involve a bridging function of vWF which binds, on one side, to components of the subendothelium such as collagen and heparin-like glycosaminoglycans and, on the other side, to the glycoprotein Ib of the platelet membrane. The interaction of vWF with glycoprotein Ib results in platelet activation[43-45] and, with the participation of other agonists, this may promote the recruitment of additional platelets into the growing thrombus. The binding of fibrinogen to glycoprotein IIb/IIIa is of major importance in this regard,[46] but vWF,[47] fibronectin,[48] and, possibly, other adhesive proteins may participate in the process by binding to the same receptor. The glycoprotein IIb/IIIa receptor may also play a role in the formation of the initial monolayer of platelets,[49] but the adhesive ligands involved have not been identified. The multimeric structure of vWF is well suited for the multiplicity of binding and bridging functions carried out by the molecule. The multivalency of the larger multimers may result in greater binding affinity for the different receptor sites and explain the greater efficacy of the larger multimers in hemostasis.

It is not presently known whether the formation of a complex between factor VIII and vWF has any consequence for the function of the procoagulant protein in addition to the protection from degradation and/or removal from the circulation.[31] vWF likely promotes the localization of factor VIII at the site of a developing clot.

VON WILLEBRAND DISEASE

The term vWD defines a bleeding disorder that is heterogeneous in its modalities of genetic transmission, clinical and laboratory manifestations, and underlying pathogenetic mechanisms. Common to the different forms of the disease is that they all represent a genetic disorder, transmitted in an autosomal manner, which alters the structure, functions, or concentration of vWF. In most subtypes, bleeding manifestations appear in heterozygous individuals, but in other cases only homozygous or doubly heterozygous subjects have evidence of disease. The underlying cause of bleeding is related to quantitative and/or qualitative abnormalities of vWF, and at least 21 different subtypes have been described based on phenotypic characteristics of the protein. Moreover, recently introduced techniques of genetic analysis have identified additional heterogeneity within subtypes of similar phenotypical characteristics.

At present, there is no systematic classification of vWD. Rather, several independent investigators have published a collection of reports describing patients with supposedly distinctive characteristics, defined by a variety of laboratory techniques. Analysis of the multimeric structure of vWF forms the basis for the differentiation between type I (all multimers present) and type II (large multimers absent), and

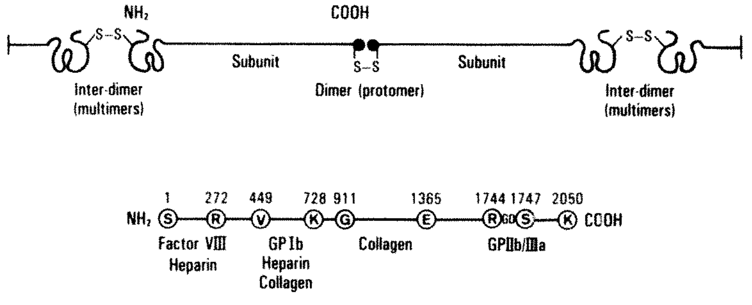

Fig 1. Schematic representation of the multimeric assembly and localization of the functional domains in vWF. Top: Two subunits form a dimer by disulfide bonding at the carboxyl termini (tail to tail). Dimers are the repeating protomeric unit of vWF multimers and are joined by covalent disulfide bonds in the amino terminal regions (head to head). Bottom: Several functional domains of vWF factor have been identified, usually by isolation of proteolytic fragments that retain a specific activity or, as in the case of the tetrapeptide Arg-Gly-Asp-Ser, by sequence analogy with other adhesive proteins. The amino and carboxyl terminal amino acid residues in each domain are indicated by one-letter abbreviation and corresponding position number in the subunit.

several distinctive patterns have been identified to date within both type I (Table 1) and type II (Table 2). The two groups, however, include patients in whom the bleeding manifestations are caused by different pathogenetic mechanisms. Patients with similar features have also been reported independently under different designations, thus adding to the confusion of the many existing subtypes.

Although we are convinced that a comprehensive classification of vWD should be proposed only after a thorough understanding of the pathophysiologic alterations of vWF structure, function, and genetic control is achieved, this may take years. Until then, it may be useful to organize the body of knowledge accumulated to date and group the patients into categories identified by well-defined parameters and presumably common pathogenetic mechanisms. This is the reason for the classification we propose. To avoid new confusing terminology, we have retained the nomenclature proposed by the authors who have originally described each new form of vWD. This attempt to rationalize the classification of vWD may be more than an exercise in futility as it may help in the choice of appropriate therapy. Indeed, because of the potential contamination with highly pathogenic viruses, the use of blood derivatives should be avoided to the extent that this is possible until safer concentrates, currently under testing, become generally available. Accurate evaluation of a patient can guide the use of 1-desamino-8-D-arginine vasopressin (DDAVP), a drug that promotes release of vWF and is very effective in many patients with vWD, but may cause thrombocytopenia in others.[52,75-79] The current guidelines for the therapy of bleeding episodes in vWD are summarized in Table 3.

Classification of vWD Based on Structural and Functional Abnormalities of vWF

In addition to recording personal and familial history and measuring the bleeding time, one should evaluate the following laboratory parameters for a comprehensive definition of patients with vWD: factor VIII procoagulant activity in plasma; vWF antigen concentration, multimeric structure and ristocetin cofactor activity in plasma and platelets; and threshold dose of ristocetin necessary to induce aggregation of platelet-rich plasma (PRP). Moreover, in selected cases, the following parameters should be evaluated: factor VIII procoagulant, vWF antigen concentration, multimeric structure, and ristocetin cofactor activity in plasma after a test

Table 1. Subtypes of Type I VWD

Designation	Reference	Distinctive Features	Comments
IA	50	All vWF multimers are present in plasma in normal relative proportion.	No evidence of intrinsic functional abnormality of vWF.
IB	50	All vWF multimers are present in plasma, but the larger ones are relatively decreased.	vWF has less ristocetin cofactor activity than normal.
IC	51	All vWF multimers are present in plasma in normal relative proportion, but a structural abnormality of individual multimers is present.	vWF has less ristocetin cofactor activity than normal.
Platelet normal	52	Platelet vWF:Ag and ristocetin cofactor activity are normal (but they are decreased in plasma).	Probably a subgroup of IA; good hemostatic response to DDAVP.
Platelet low	52	Platelet vWF:Ag and ristocetin cofactor activity are equally decreased (as in plasma).	Probably a subgroup of IA; poor hemostatic response to DDAVP.
Platelet discordant	52	Platelet vWF:Ag is normal but ristocetin cofactor activity is decreased (both decreased in plasma; discrepancy becomes evident in plasma after DDAVP).	Probably corresponds to IB; poor hemostatic response to DDAVP.
I-1	53	vWF:Ag is decreased in plasma and platelets.	Similar to platelet low.
I-2	53	vWF:Ag is decreased in plasma but normal in platelets.	Similar to platelet normal.
I-3	53	Normal concentration of vWF:Ag in plasma, but decreased in platelets.	One of the two patients reported was the clinically unaffected parent of a patient with severe vWD.
I, New York	54	Platelet-rich plasma shows aggregation at ristocetin concentrations lower than necessary for normal; all vWF multimers are present in plasma.	Hyperresponsiveness to ristocetin similar to type IIB, but larger multimers are absent in the latter.
Undesignated	55	Similar to the description above.	Similar to type I, New York.
Undesignated	20	Pro-vWF is present uncleaved in vWF multimers (subunit larger than normal).	Only one family studied.
Undesignated	56	A group of patients has been identified in whom multimers larger than normal are present in plasma under baseline conditions.	The baseline multimeric pattern is like that of normal individuals after infusion of DDAVP.

Abbreviation: vWF:Ag, von Willebrand factor antigen.

Table 2. Subtypes of Type II vWD

Designation	Reference	Distinctive Features	Comments
IIA	57-59	Large and intermediate vWF multimers are absent in plasma and platelets.	Increased proteolysis of vWF; some variability in the size of multimers present. Heterogeneous response to DDAVP; few cases show recessive inheritance.
IIA-1	53	Normal concentration of plasma and platelet vWF:Ag; abnormal multimeric structure.	Single case reported.
IIA-2	53	Decreased concentration of plasma and platelet vWF:Ag; abnormal multimeric structure.	Single case reported.
IIA-3	53	Decreased concentration of plasma vWF:Ag, but normal in platelets; abnormal multimeric structure in plasma, but normal in platelets.	
IIB	57,60,61	Hyperresponsiveness to low doses of ristocetin; large vWF multimers are absent in plasma; all multimers are present in platelets.	Increased proteolysis of vWF; thrombocytopenia following DDAVP; few cases show recessive inheritance.
Undesignated	62	Thrombocytopenia; spontaneous platelet aggregation; abnormal multimeric structure.	Probably a different phenotypic manifestation of subtype IIB vWD. Also reported as type IIB Tampa.[62a]
IIC	63-67	Large vWF multimers are absent; unique structural abnormality of individual multimers. Recessive inheritance (possible heterozygosity).	Decreased proteolysis of vWF; some heterogeneity in the multimeric pattern.
IID	68,69	Large vWF multimers are absent; unique structural abnormality of individual multimers.	Decreased proteolysis of vWF.
IIE	70	Large vWF multimers are markedly decreased; unique structural abnormality of individual multimers.	Decreased proteolysis of vWF.
IIF	71	Large vWF multimers are absent in plasma, but present in platelets; unique structural abnormality of individual multimers.	Single case reported; modality of genetic transmission unclear.
IIG	72	Probably dominant inheritance; unique structural abnormality of individual multimers.	Single case reported (both parents deceased).
IIH	73	Recessive inheritance; unique structural abnormality of individual multimers.	Single case reported.
Type B	74	Normal multimeric structure, but abnormal crossed immunoelectrophoresis (two peaks); no response to ristocetin, but normal to botrocetin.	Single case reported.

infusion of DDAVP; multimeric structure of vWF with different gel systems to detect intrinsic structural abnormalities of individual multimers; and response of PRP to botrocetin. Patients with vWD may be grouped in the following categories.

Patients with quantitative abnormalities and no evidence of intrinsic functional abnormality of vWF. Patients with quantitative abnormalities and no evidence of intrinsic functional abnormality of vWF may include the majority of individuals with vWD (≥50%), corresponding to type IA with the subtypes "platelet normal" (I-2), "platelet low" (I-1), I-3, the variant with persistence of pro-vWF, and the variant with larger than normal multimers. The genetic transmission of the disease is, with a few exceptions, dominant. Most cases present with low plasma levels of vWF antigen, usually between 5% and 30% of normal, and correspondingly low levels of ristocetin cofactor activity (the assay reflecting the property of vWF to bind to glycoprotein Ib and mediate platelet agglutination). The factor VIII procoagulant protein is also decreased in proportion to the decrease of vWF. The pathogenesis of bleeding, in these cases, is due to insufficient levels of circulating vWF and factor VIII. Bleeding manifestations are less severe in individuals who have a normal concentration of platelet vWF. The latter patients usually have excellent hemostatic response to DDAVP; those with low platelet concentration of vWF may have a poor response.

Patients whose vWF has low ristocetin cofactor activity. With the possible exception of the patient designated as type B, all subtypes listed in this category exhibit abnormal multimeric structure of vWF with decrease or absence of the larger multimers. The ristocetin cofactor activity is always lower than the levels of vWF antigen, which can be decreased or normal. The levels of factor VIII procoagulant are usually proportional to the levels of vWF antigen and may therefore be normal or decreased. The group includes types IB (plate-

Table 3. Guidelines for the use of DDAVP in Patients With vWD

DDAVP Usually Effective	DDAVP Usually Ineffective*	DDAVP Is Ineffective	DDAVP Contraindicated	Insufficient Data
Type IA (normal platelet content of vWF)	Type IA with low platelet content of vWF	Type III	Type IIB	Type I, New York, and similar
Undesignated variant with larger than normal multimers	Type IB (platelet discordant)			
Undesignated variant with persistence of pro-von Willebrand factor	All subtypes of type II		Pseudo-vWD or platelet-type vWD†	
Type IC				

*The increased levels of factor VIII procoagulant following DDAVP infusion may help control bleeding in certain circumstances, despite the persistent abnormality of vWF function. Moreover, favorable effects on vWF function have been reported in some cases and, therefore, a trial infusion with monitoring of the bleeding time and multimeric composition of vWF is indicated.

†Normal vWF causes platelet agglutination and thrombocytopenia in these patients. DDAVP and vWF concentrates therefore may have the same effect.

let discordant), IC, IIA (with its subtypes IIA-1, IIA-2 and IIA-3), IIC, IID, IIE, IIF, IIG, IIH, and type B. Some of the typical structural abnormalities of vWF that occur in these subtypes are shown in Fig. 2.

The pathogenesis of bleeding is believed to be related to the absence of the larger multimeric forms of vWF, secondary to abnormal synthesis. Evidence of increased proteolysis of the larger multimers in some patients is also likely to be the reflection of intrinsic abnormalities of the vWF molecule. Bleeding may be more severe in patients who have decreased concentrations of both vWF and factor VIII. The hemostatic response to DDAVP is poor in most patients in this group, but may be sufficient in some individuals with type IB and IIA (presumably those with a less abnormal structure and normal levels of platelet vWF). Therefore, to minimize the risk of potentially severe diseases transmitted with blood products, the effect on the bleeding time of a trial infusion of DDAVP should be evaluated in all these patients. This is reasonable also in view of the minimal side effects of the drug.

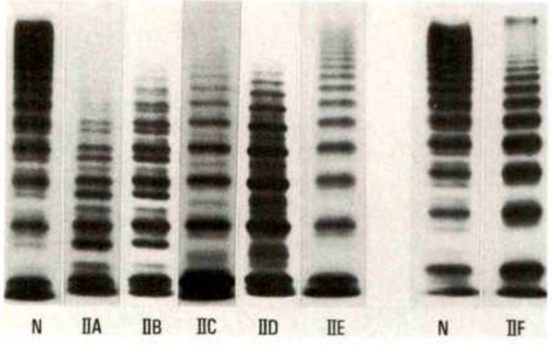

Fig 2. Multimeric structure of vWF in subtypes of type II vWD. Presented are the subtypes that we have had an opportunity to study to date. Multimeric analysis was performed in agarose gels containing sodium dodecyl sulfate, as previously described.[63] N indicates normal control plasma. The six lanes on the left and the two lanes on the right are derived from different gels. Samples were applied at the top of the gel (cathode), and the anode was at the bottom. vWF multimers were identified by reaction with ^{125}I-labeled monospecific anti-vWF antibodies. Common to all the subtypes of type II vWD is the lack (or very marked decrease) of larger vWF multimers (those moving closer to the top of the gel). In the gel system used, each multimer of normal vWF shows three bands. In subtypes IIA and IIB, these bands have the same electrophoretic mobility as in normal plasma, but their relative intensity is different and the faster moving band in each triplet exhibits increased intensity. More profound derangements of this structure, in the form of lack of certain bands or appearance of bands with abnormal mobility, or both, are evident in subtypes IIC, IID, IIE, and IIF. The structural abnormalities that occur in the subtypes of type II are likely to reflect alterations in the primary structure of vWF that are as yet unknown. Sequence abnormalities may result in structural abnormalities either directly or because they change the susceptibility of the protein to proteolytic degradation.[58,70]

Patients with enhanced responsiveness to ristocetin. In patients with enhanced responsiveness to ristocetin, PRP shows aggregation at concentrations of ristocetin lower than those necessary in normal individuals. The group includes type I-New York (and the similar variant described by Holmberg et al) and type IIB. Of particular interest are the patients (all type IIB) who manifest transient or persistent thrombocytopenia. The episodes may occur with no apparent relation to other events or be associated with conditions such as stress, physical effort, or pregnancy, which cause increased plasma levels of vWF. Thrombocytopenia also appears following infusion of DDAVP.[77] These patients synthesize an abnormal vWF molecule that, unlike the normal counterpart, is capable of interacting directly with circulating platelets, presumably at the glycoprotein Ib site, causing platelet aggregation with consequent removal from the circulation.[44] It is apparently contradictory that patients whose vWF binds with higher affinity to glycoprotein Ib have bleeding rather than thrombotic manifestations. We interpret this as evidence that the physiologic sequence of events initially involves the interaction of vWF with the subendothelium and then with platelets. Blocking of the glycoprotein Ib receptor on circulating platelets prevents their anchoring to vWF bound to the subendothelium. Most patients in this group can be identified with those described as type IIB vWD. They characteristically show absence of large vWF multimers from plasma, presumably as a consequence of their interaction with platelets. Patients with the other variant forms of vWD, showing enhanced response to ristocetin but normal multimeric structure of vWF, are not known to manifest thrombocytopenia, either spontaneous or following DDAVP infusion.

Other patients have been described who exhibit structural abnormalities of vWF and enhanced responsiveness to ristocetin, as well as episodes of thrombocytopenia, like those of patients with type IIB vWD, but they represent a distinct group in that they have an intrinsic platelet abnormality. In these cases, the vWF abnormalities are secondary to the interaction with abnormal platelets. These individuals have been included in a group called pseudo-vWD[80] or platelet-type vWD[81,82] (presumably the same disease, described independently by two laboratories).

The therapy of bleeding episodes in patients with type IIB vWD is based on use of blood derivatives.[76] DDAVP is considered contraindicated because it causes acute thrombocytopenia or worsens existing thrombocytopenia,[77] presumably as a consequence of increased release of the abnormal vWF, which induces platelet aggregation. Nevertheless, DDAVP does not seem to cause vascular occlusions in the microcirculation. The drug has no beneficial effect on the bleeding time in type IIB patients,[83] although it raises the levels of factor VIII and vWF.

In the case of pseudo- or platelet-type vWD, the infusion of cryoprecipitate, particularly a high dose,[84] may result in thrombocytopenia. This is believed to be secondary to the increased plasma levels of vWF interacting with the abnormal platelets of the patients[82] and may be expected to happen even after infusion of DDAVP. It is difficult to give guidelines of general value for treatment of these cases, although a combination of normal platelet concentrates and low-dose vWF concentrate in cases with severe bleeding appears reasonable. Moreover, the response of different patients to the increased plasma levels of vWF varies considerably, and this may affect the dose of concentrate that an individual patient can tolerate before developing thrombocytopenia.

Patients with type III (severe) vWD. Patients with the so-called "severe" form of vWD (defined by some as severe type I or, by others, type III) are likely to manifest major bleeding episodes from early in life. We believe that they represent a separate group (and not a severe manifestation of type I) because of the typically recessive modality of genetic transmission and because in some cases the trace amounts of vWF present in plasma and platelets may show evidence of structural abnormalities.[85] Children with severe vWD are typically born to asymptomatic parents and have extremely low levels of vWF, usually <1% of normal.[86] As a consequence, the levels of factor VIII, the procoagulant protein, are also very low, although usually measurable between 2% and 10% of normal. Symptoms similar to those of persons with hemophilia, including hemarthroses and muscular hematomata, are not unusual, and mucosal bleeding is very severe in most patients. The bleeding time is invariably very prolonged.

The genetic abnormalities responsible for severe vWD have been recently investigated using cDNA probes. These studies have led to the demonstration of complete or partial deletions of the translated regions of the vWF gene. In some patients, both chromosomes are affected (homozygous patients); the asymptomatic parents are heterozygous for the same deletion.[87,88] The synthesis of vWF is completely suppressed in the patients with homozygous deletions and, as a consequence, heteroantibodies develop after treatment with blood derivatives containing vWF, which in these individuals, represents a foreign antigen.[89-91] In other cases, complete deletions of the translated regions of the vWF gene have been demonstrated, but only one chromosome is affected, as judged by DNA dosage.[88] In these cases, the disease is probably due to the concomitant inheritance of another, yet undefined, heterozygous abnormality. These patients, therefore, are doubly heterozygous for two distinct abnormalities, and their parents are heterozygous for each of them. A third group of patients shows no detectable alterations of the vWF gene, at least as demonstrated by using cDNA probes, and it is not now possible to establish whether they are homozygous or doubly heterozygous.[87,88] Appearance of antibodies against vWF is unlikely in the latter two groups of patients.

The treatment of patients with severe vWD requires infusion of sufficient amounts of blood derivatives containing factor VIII procoagulant activity and functional vWF. The same applies before any surgical intervention. Treatment with DDAVP is not useful in these individuals because the endothelial cells of the patients cannot synthesize significant amounts of vWF.

CONCLUSIONS

The many subtypes of congenital vWD described to date present a confusing picture. It is our hope that this review has helped the reader achieve a better understanding of this topic. The knowledge gained in studying congenital vWD may also be useful for a better understanding of acquired alterations of vWF. These alterations may include decreased plasma levels as well as structural and functional abnormalities and may be encountered in a variety of pathologic conditions or in otherwise healthy individuals.[92-99] Immunologic mechanisms have been implicated in some instances, whereas in others proteolysis appears to play a role. Proteolysis appears to destroy large multimers preferentially and may be operative in the acquired forms of vWD that sometimes occur in conjunction with essential thrombocythemia,[100] congenital cardiac defects[101], and chronic myelocytic leukemia.[102] However, no satisfactory explanation has been offered for the vWF abnormalities in a number of instances of acquired vWD.

vWF may participate in a variety of pathologic processes by virtue of its prothrombotic attributes. Unusually large multimers of vWF have been identified in the circulation during the course of thrombotic thrombocytopenic purpura and have been postulated as playing an etiologic role in the devastating occlusive events that occur in the microcirculation in this disorder.[103] Because of the likely function of vWF in promoting platelet adhesion to perturbed endothelial surfaces, this protein may be an important pathophysiologic factor in the development of atherosclerotic lesions,[104-106] but the mechanism(s) involved are poorly understood at present. As our understanding of the pathophysiologic role of vWF progresses, it is to be anticipated that modalities will be developed to regulate its function in health and disease.

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REVIEW

Radiogenic Leukemia Revisited

By William C. Moloney

Radiation-induced leukemia is considered to be similar to the de novo disease. However, following an analysis of clinical and hematological findings in leukemia occurring in irradiated cervical cancer patients, adult Japanese atomic-bomb survivors, and spondylitics treated with x-ray, striking differences were noted. Acute leukemias in cervical cancer patients and Japanese survivors were similar in type to acute de novo leukemias in adults. Cell types among spondylitics were very dissimilar; rare forms, eg, acute erythromyelocytic leukemia (AEL) and acute megakaryocytic leukemia, were increased. Pancytopenia occurred in 25 of 35 cases and erythromyelodysplastic disorders were noted in seven of 35 acute cases. The leukemias and myelodysplastic disorders closely resembled those occurring in patients treated with alkylating agents. This similarity suggests a common pathogenesis involving marrow stem cell injury and extra-medullary mediators of hematopoiesis. Investigation of early acute leukemias and myelodysplastic disorders with newer techniques may provide valuable insights into the pathogenesis of leukemia in humans.

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THE LEUKEMOGENIC effect of ionizing radiation was first described by von Jagic et al in 1911.[1] Since then many individual case reports and small groups of leukemias attributed to radiation exposure have been reported. However, only three large series, well-studied epidemiologically and hematologically, have been published. The latter include cases among Japanese atomic-bomb survivors,[2] leukemia occurring in British spondylitics treated with x-ray[5] and leukemia in women with cervical cancer following radium and x-ray therapy.[4] It is generally accepted that the types of leukemia induced by radiation are similar to the de novo disease; however, when the clinical and hematological findings from the three series mentioned above were reviewed, interesting differences were noted. These observations and their possible implications for the role of ionizing radiation in the pathogenesis of leukemia are presented in this report.

MATERIALS AND METHODS

Atomic-bomb survivors. In 1947 the US government established the Atomic Bomb Casualty Commission (ABCC), and in 1950 set up field headquarters in Hiroshima and Nagasaki. Extensive studies were carried out on survivors in order to determine the late effects of radiation. In 1975 the Japanese government assumed responsibility for these investigations under the auspices of the Radiation Effects Research Foundation (RERF). Numerous articles and reports have been published, many concerned with the incidence and dose relationship of radiation-induced leukemia.[2] In an overall review Ichimaru et al[3] published an article on 149 cases of definite and probable leukemias occurring from November 1945 to October 1971 among 82,000 atomic-bomb survivors and 27,000 others not in the cities at the time of bombing. All leukemias in these survivors, including 13 that occurred before October 1, 1950, were listed; other data included age at time of bombing, type of leukemia, date of onset, and estimated radiation dose. In the present analysis records on 98 of these Japanese patients over the age of 30 were reviewed. In order to obtain more detailed information on the types of leukemia and to determine whether a preleukemic stage occurred, abstracts of case histories and reports on peripheral blood and bone marrow studies along with results of autopsies were examined on the 78 Hiroshima and 20 Nagasaki survivors. To carry out a comparative analysis the following changes were made in nomenclature used by Ichimaru et al; acute stem cell leukemia (ASL) to acute unclassified leukemia (AUL), acute myelogenous leukemia (AML) to acute monocytic leukemia (AMOL), and erythroleukemia to acute erythromyelocytic leukemia (AEL). Ichimaru et al did not identify cases of acute monomyelocytic leukemia (AMML) and these cases were apparently included with AMOL. It should be noted that no cases of acute progranulocytic leukemia (APL) or acute megakaryocytic leukemia were reported among these atomic-bomb survivors. As far as possible acute leukemias were reclassified according to the French, American, and British Nomenclature Committee (FAB).[4]

Spondylitic patients treated with x-ray. A well-documented series of leukemias was reported in 1957 by Court Brown and Doll[5] among 13,352 irradiated spondylitics. To be accepted for that study the diagnosis of spondylitis had to be confirmed, amount and duration of x-ray therapy established, and only cases occurring 12 months or more following x-ray therapy were included. In the Court Brown and Doll study, intensive efforts were made to identify and observe all cases, and detailed clinical, hematological, and pathological data were published in their monograph. In view of the changing myelopathic concepts and difficulties in classifying disorders, an attempt was made to reevaluate 57 cases from the original Court Brown and Doll series. Case histories were reviewed along with available peripheral blood and bone marrow specimens as well as postmortem results. Radiation doses were obtained from the case histories. Twelve cases were omitted for a variety of reasons, and as far as possible the leukemias and other myelopathic disorders were reclassified according to the FAB nomenclature.

Leukemia occurring in women following radiation therapy for cervical cancer. Beginning in 1960, an investigation was carried out on the incidence of leukemia and other malignancies occurring in women with cervical cancer treated with radium to the cervix and x-ray to the lower abdomen. This study was under the auspices of the International Agency for Research on Cancer (WHO) and the Radiation Epidemiology Branch, National Cancer Institute. In a recent article describing this study Boice et al[5] reported that 182,616 patients had been enrolled in the program: of these, 82,616 had invasive cervical cancer requiring radiation therapy. At the time of the report the average period of observation was 7.6 years with a total of 603,788 women-years of follow-up. Intensive efforts were made to obtain accurate assessment of radiation dosage: average dose to the active marrow was 300 to 1,500 rads. Considerable care was taken to verify the diagnosis and type of leukemia. Due to

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excellent cooperation of the tumor registries and clinics involved in the study, clinical information along with results of blood and bone marrow examinations were obtained in over 80% of cases. When the diagnosis was in doubt, marrow specimens were submitted for central review. When slides could not be sent out of the country, opinions were obtained from local experts. As far as possible the acute leukemias were reclassified according to the FAB system.

Leukemia in adults de novo. As part of this comparative analysis a series of leukemia patients observed in the Hematology Division, Brigham and Women's Hospital, from 1969 to 1979 were reviewed. Only patients 30 years of age or older were selected for this study and individuals with a prior history of refractory anemia, multiple myeloma, non-Hodgkin's lymphoma, Hodgkin's disease, or myeloproliferative disorders were excluded. No patient had received chemotherapy or significant radiation exposure before the onset of leukemia. There were 232 cases of acute leukemia, 94 females and 138 males, as well as 86 cases of chronic myelogenous leukemia (CML). The acute leukemias were classified according to the FAB classification.

RESULTS

CML. Among a total of 98 cases of leukemia in Japanese survivors, 37 were CML (18 females, 19 males). CML was typical hematologically and clinically. Median age was 50 with a range of 31 to 84 years. Median latent period was 11 years (range 2 to 25 years). The median radiation dose was 45 rads with a range of 1 to 443 rads. There was no estimate of dose in four cases and zero dose was recorded in four cases. It is of interest to note that of 20 leukemias in Nagasaki only three were CML, while in Hiroshima 34 of 78 cases were CML.

Among the 43 leukemias in the irradiated spondylitic patients there were eight cases of CML (seven males, one female). The average age was 45 years with a range of 30 to 64. While the small number precludes a meaningful analysis, it is noteworthy that the latent periods were considerably shorter than in the Japanese survivors or cervical cancer patients: average 3.6 years with a range of one to six years. Radiation dose was available in seven cases with an average of 1,708 rads and a range of 377 to 2,706 rads.

Of the 139 cases of leukemia in the irradiated cervical cancer patients 40 were CML. Clinical and hematological features were typical of CML. Median age was 58 (range of 41 to 83 years) and the median latent period was 9 years (range 1 to 23 years). The median dose of radiation to the bone marrow was 750 rads (range 300 to 1,500 rads).

Acute leukemias and myelodysplastic disorders. Among Japanese survivors 61 cases of acute leukemia over age 30 were reviewed (29 females and 32 males). The median age was 54 (range 30 to 78 years), the median radiation dose was 120 rads (range 1 to 900 rads). Median latent period was 17 years (range 6 to 31 years). Following my review of the clinical histories, peripheral blood, bone marrow, and postmortem findings, cases were reclassified as follows: acute granulocytic leukemia (AGL) (M1 and M2) 29, AMML (M4) seven, AMOL (M5) seven, AEL (M6) three, ALL ten, AUL five. In general the acute leukemias resembled de novo cases; pancytopenia was present in four cases; however, blast cells were found either in the peripheral blood or bone marrow. In two of the three cases of AEL, pancytopenia was present at time of diagnosis and ring sideroblasts were noted in the bone marrow.

Among the irradiated spondylitics, there were 35 cases (34 male, one female) designated as AML by Court Brown and Doll, and, in addition, two cases of aplastic anemia proven by biopsy and autopsy. Median age was 41 (range 22 to 70 years). Estimated latent period (using a mean between first and last x-ray therapy) gave a median of 3.0 and a range of 1 to 12 years. Median dose of radiation was 1,070 rads with a range of 220 to 3,525 rads. Twenty-five cases were pancytopenic at onset of the myelopathic disorder; in one case no leukocyte count was available. For all 35 cases median leukocyte count was 3,400/mm^3 (range 700 to 240,000). In the peripheral blood smears leukemic cells were noted in 15, none were present in 18, and in two cases peripheral smears were not available. Following a review of the clinical histories, blood studies, bone marrow aspirates and biopsies, as well as results of postmortem examinations, the eight cases of AGL could be separated into M1 and M2 from descriptions of myeloblasts and promyelocytes in blood smears and bone marrow specimens. There were seven cases of AMOL in the Court Brown and Doll report, a diagnosis based on morphological criteria. The designation AMML was not widely used 30 years ago, but no doubt cases of this type were included in the AMOL category. A diagnosis of ALL was made in two cases and AUL in three. In three cases, while acute leukemia was apparently present, the cell type could not be established due to lack of adequate blood and marrow specimens.

An unusual feature of the hematological disorders in this series was the presence of nine patients with erythromyelocytic abnormalities in the marrow. These are included in the Court Brown and Doll report as AML–aleukemic. In two cases the marrow findings strongly suggest to this investigator a diagnosis of AEL and in two others either refractory dysmyelopoietic anemia (RDA) or refractory anemia with excessive blasts (RAEB). There were five patients who had marked erythroid proliferation associated with the presence of promyelocytes and myeloblasts; these marrow findings are consistent with a diagnosis of transition from RAEB to AML (RAEB-T).

Three other cases with an initial diagnosis of AML are of considerable interest. All three had features at bone marrow biopsy and autopsy of myelofibrosis and were termed acute myelofibrosis or myelosclerosis. In the light of recent technical developments in hematology and pathology, these cases appear to be candidates for the diagnosis of acute megakaryocytic leukemia, a diagnosis proposed in two of three cases by the pathologist who originally reviewed the postmortem material for the Court Brown and Doll report.[7]

The cervical cancer study provided excellent clinical and hematological information in the great majority of the 99 cases of acute leukemias. In this group the median age was 65 (range 38 to 89), latent period, median eight years (range 1 to 41). The median radiation dose was 720 rads (range 210 to 2,000 rads). After review, 52 cases were classified as AGL (M1 and M2) consisting of 37.4% of the acute leukemias, a figure similar to that found in the de novo series (see Table 1). The remaining cases consisted of APL three, AMML seven, AMOL seven, AEL three, AUL ten, ALL 16 and one

Table 1. Classification of Leukemia in 3 Irradiated and 1 De Novo Population

Diagnoses	Cervical Cancer	A-Bomb Survivors	Spondylitics	De Novo
CML	40 (28 7)	37 (37.7)	8 (18 4)	86
AL/MDS	99	61	35	241
M1 + M2	52 (52.5)	29 (45.9)	8 (22.8)	101 (41.9)
M3	3 (3.0)	0 (0)	0 (0)	13 (5.4)
M4	7 (7.0)	7 (11.6)	0 (0)	37 (15.4)
M5	3 (7.0)	7 (11.6)	7 (20.0)	26 (10.8)
M6	3 (3.0)	3 (4.9)	2 (5.7)	11 (4.6)
M7	1 (1.0)	0 (0)	3 (8.3)	9 (3.7)
AUL	10 (10.0)	5 (8.2)	3 (8.3)	9 (3.7)
ALL	16 (16.0)	10 (16.3)	2 (5 7)	18 (7.5)
RDA	0	0	1 (2.8)	—
RAEB	0	0	1 (2.8)	—
RAEB-T	0	0	5 (14.2)	—
? AML	0	0	3 (8 3)	17 (7.1)
Total	139	98	43	327

Abbreviations: CML, chronic myelogenous leukemia; AL, acute leukemia; MDS, myelodysplastic syndrome; ALL, acute lymphatic leukemia

case of acute megakaryocytic leukemia. In two cases of AEL and one AGL, ringed sideroblasts were present in the bone marrow. In one case of AUL there was a six-month history of pancytopenia before the diagnosis of leukemia.

The distribution of types of acute leukemia in the de novo series may be noted in Table 1. Since the de novo series consisted of patients referred to the Hematology Division, Brigham and Women's Hospital, it may not necessarily be fully representative of all de novo adult leukemias. Special studies, including histochemical stains and immunological markers, were carried out on many of the problem cases. This may account for the relatively low incidence of AUL and ALL cases in this series.

DISCUSSION

Although it is generally assumed that radiogenic leukemia is similar to the de novo disease, it is evident from the observations in this review that marked differences exist between irradiated spondylitics compared with Japanese A-bomb survivors, women irradiated for cervical cancer, and de novo acute leukemia patients. In all three series of irradiated patients CML was typical clinically and hematologically. However, considerably fewer cases occurred in spondylitics (8) than among Japanese survivors (37) or the cervical cancer patients (40) (Table 1). The elevated incidence in Japanese survivors involved only Hiroshima; of 37 cases, 34 occurred in Hiroshima and only three in Nagasaki. The increased incidence in Hiroshima has long been recognized, but to date no suitable explanation has been forthcoming.[8] The average latent period of CML in spondylitics was 3.5 years compared with a median latent period of 9.0 years for cervical cancer patients and 11 years for Japanese survivors.

In all types of acute leukemia clinical and hematological features were similar in Japanese survivors, cervical cancer patients, and the de novo series. However, these three groups pose a striking contrast to acute leukemias and related disorders among irradiated spondylitics. In the latter series 25 of 35 cases, collectively classified as AML in the Court Brown and Doll report, were pancytopenic at onset and required bone marrow examination to establish a diagnosis. The spectrum of myelopathic states included nine with striking erythromyelodysplastic features. The presence of a pancytopenic stage accompanied by lack of leukemic cells in the peripheral blood bears a close resemblance to features of the acute leukemias and myelodysplastic states following treatment with alkylating agents. Similar also are the unusual cell types, eg, erythroleukemia and acute megakaryocytic leukemia. Other shared features are the rapidly fatal course and lack of response to chemotherapy. Unfortunately, the leukemias among spondylitics were encountered before 1958 and no cytogenetic studies were available.

In the past 15 years there has been an increasing recognition of so called "preleukemia" states, chiefly refractory dysmyelopoietic anemia with or without ringed sideroblasts in the bone marrow.[9,10] Many studies have been carried out on both the de novo disease and those secondary to chemotherapy in order to clarify the relationship of these disorders to acute leukemia. The FAB established a classification for acute leukemias that provides a useful means of comparison for hematologists and other interested groups.[11] The designations of RDA, RAEB, and the more controversial refractory anemia with excess blasts in RAEB-T, have provided additional descriptive terminology. Along with the newer morphological developments, the recognition of clonality and nonrandom chromosomal abnormalities in the myelodysplastic states, especially 5−, 5q−, 7−, 8+, and 13−, has added new dimensions to the diagnosis and investigation of these disorders and their relationship to AML.[12]

To account for the unique effect of x-ray therapy in spondylitics two possibilities should be considered: (1) that patients with spondylitis have a predisposition for developing leukemia; or (2) the x-ray therapy used had special leukemogenic characteristics. Ankylosing spondylitis is an inflammatory arthritis but as far as is known carries no increased risk of malignancy.[13] Moreover, in addition to direct injury to marrow stem cells, the possibility should be considered that spondylitics may have defective T lymphocytes or T cells that are unduly radiosensitive.[14,15] X-ray therapy in spondylitics was administered in one, or more often, in a number of courses. It was delivered in relatively high dosage over large joints and the spine, areas containing major amounts of bone marrow. Furthermore, orthovoltage x-rays interacting with dense bone may release photons that can deliver high levels of ionizing radiation to local areas, especially the endosteum, stromal cells, and marrow stem cells contiguous to the endosteal lining. Recently considerable attention has been directed to the relationship of stromal cell damage and leukemogenesis.[16-18] The studies by Greenberger and his co-workers on the isolation of a new factor capable of transforming normal mouse marrow cells into malignant myeloblasts is of special interest in this regard. In considering the pathogenesis of radiation-induced leukemia it is important to emphasize that the acute leukemias and myelodysplastic disorders following therapy with alkylating agents closely resemble, both clinically and hematologically, the disorders occurring in irradiated spondylitics. Since both modalities of therapy damage stem cells and also suppress or modify lymphocytes, which mediate hematopoiesis, it is reasonable to postulate that leukemias and myelodysplastic disorders may be caused by a combination of these effects.

In spite of intensive research for the past 70 years, the role of radiation in leukemogenesis remains an enigma. However, recent developments in the field of molecular biology, especially the discovery of oncogenes,[19,20] studies on clonality and differentiation of myeloblasts such as those carried out by Fialkow,[21] and the investigation of the differentiation of human myeloblasts with the use of DNA probes by Fearon et al give promise of important progress in the field of leukemogenesis.[22] Application of these newer techniques to the myelodysplastic disorders and early phases of leukemia secondary to radiation and alkylating agents present unique opportunities for the type of research that may lead to better understanding of the pathogenesis of leukemia in man.

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Genetic Differences in Hemoglobin Influence on Erythrocyte Oxidative Stress Hemolysis

By Walter C. Kruckeberg, David I. Doorenbos, and Priscilla O. Brown

The RBC from mice of certain inbred strains hemolyzed under oxidative stress (2.0 mmol/L hydrogen peroxide), whereas red cells from mice of other strains did not. In the experimental system human erythrocytes did not hemolyze. The rate of formation of malonyldialdehyde (a fatty acid oxidative breakdown product) was fourfold higher in hemolytic *v* nonhemolytic red cells. There was insufficient variation in the levels of glutathione peroxidase activity or its substrate, reduced glutathione, to explain these hemolysis differences. On the other hand, the antioxidants butylated hydroxyanisole and hydroxytoluene, and histidine protected the hemolysis-prone red cells from breaking open. The hemolysis trait demonstrated autosomal recessive Mendelian inheritance. When using inbred, recombinant inbred, and congenic inbred mice, this hemolysis/nonhemolysis trait correlated 1:1 with the type of hemoglobin β chain in the RBC. This experimental system is a potential model for investigating the role of hemoglobin in prehemolytic events.

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TOXIC OXIDANT RADICALS have been implicated in RBC hemolysis in a number of congenital or acquired conditions including β-thalassemia major,[1] sickle cell anemia,[2] unstable hemoglobin (Hb),[3] enzyme deficiencies,[4-7] and aging of RBCs.[8]

There are numerous intracellular events that precede oxidant-induced cell hemolysis. These include oxidation of Hb,[9] generation of high-molecular weight proteins or protein fragments,[10,11] ionic shifts,[12] lipid peroxidation,[13] changes in cell deformability,[14] and phospholipid flip-flop within the lipid bilayer leaflet of the membrane.[15] It is still uncertain which of these changes are prerequisite to hemolysis and in what order the events occur. It is also important to clarify the specific role that Hb plays in prehemolytic events.

As reported here, RBC from mice of certain inbred strains withstand oxidative (hydrogen peroxide) stress and do not hemolyze, whereas red cells from mice of other strains hemolyze under the same conditions. This trait of hemolysis/nonhemolysis follows the roles of Mendelian inheritance and is apparently determined by the type of Hb β chain carried within the cells. This experimental system represents a genetic model for studying the role of Hb in prehemolytic events during oxidant stress.

METHODS AND MATERIALS

Cell preparation and assays. Adult mice were anesthetized with ether and bled by cardiac puncture into syringes containing 50 units of sodium heparin. The cells were washed by centrifugation (12,000 g, five minutes, 4°C) in 0.008 mol/L NaPO$_4$ (pH 7.4), 0.131 mol/L NaCl, 0.008 mol/L KCl, and 0.004 mol/L NaN$_3$. The washed red cells were suspended to a 10% hematocrit in the same buffer and 0.9 mL preincubated for ten minutes in a 37°C shaking water bath. At that point 0.1 mL of 0.02 mol/L hydrogen peroxide in isotonic saline was added to the cell suspension with immediate mixing. The concentration of hydrogen peroxide in the commercial stock was determined by using the molar extinction coefficient of 71 at 230 nm.[16] At timed intervals, aliquots were withdrawn from the incubation mixture and centrifuged (12,000 g, five minutes, 4°C). Supernatant Hb concentrations were then determined[17] as the assay for hemolysis. Alternatively, uncentrifuged aliquots were assayed for malonyldialdehyde.[18] The chemicals used were the purest available from Sigma Chemical Co, St Louis.

Glutathione peroxidase assay The assay for maximum glutathione peroxidase activity[17] consisted of 0.2 mL of 0.2 mol/L Tris-HCl, 0.001 mol/L EDTA, (pH 8.0), 0.08 mL of 0.1 mol/L reduced glutathione (GSH), 0.5 mL of 10 U/mL glutathione reductase (GR), 0.3 mL of 0.002 mol/L NADPH, 0.02 mL hemolysate, 0.1 mL of 0.034 mol/L *t*-butyl hydroperoxide (tBH), and 1.8 mL deionized water. The assay was monitored at 340 nm at 37°C and was initiated by adding the hemolysate to the prewarmed constituents in a 3.0-mL cuvette. Blank assays (ie, water instead of hemolysate added to the reaction mixture) were run with each set and the optical density substracted from that of the complete reaction mixture. If the GSH, GR, or tBH was left out of the reaction mixture, there was essentially no change in optical density.

The enzyme reaction rate was tested in hemolysates made from whole blood and from blood that had been filtered to remove WBCs.[17] The presence or absence of white cells had no effect on the rate of enzyme activity (unpublished data)

Assay hemolysates were made by diluting 0.1 mL hyparinized whole mouse blood in 0.9 mL deionized water. After one minute, 2.0 mL of 0.2 Mol/L Tris-HCl and 0.001 mol/L EDTA (pH 8.0) was added and the hemolysate frozen, thawed, and assayed.

GSH assay. The levels of GSH in the mouse RBCs were assayed by using the method of Beutler.[17]

Mouse Hb β chain There are three codominant haplotypes that control mouse Hb β chains: a p-diffuse genotype (signified with the letter *p*) and the more common single (s) and diffuse (d) genotypes that are named after, respectively, single and diffuse Hb patterns after electrophoresis.[19] The genes for p-diffuse, single, and diffuse Hb β chains are signified, respectively, Hbb^{p}, Hbb^{s}, and Hbb^{d}. The Hbb^{d} loci contain two linked genes that produce structurally different Hb. The single Hb β chain haplotype encodes one β chain structurally different from the diffuse and p-diffuse Hb β chains.[20,21]

Sources of mice. Mice of inbred strains A, AU/Ss, BALB/c, BALB/cBy, C3H/He, C57BL/6, and DBA/2 as well as seven recombinant inbred strains of CXB were obtained from the Jackson Laboratory, Bar Harbor, ME Mice of inbred strains AKR, CBA, C3H/Fg, C57BL/10Sc, C57L, NZW, ST/b, and STR/1 were obtained from the National Institutes of Health, Bethesda, MD.

From the University of Mississippi Medical Center, Jackson.

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Mice of congenic strains C57BL/6J.*Mus castaneus*-Hbb^d, C57BL/6J.Peru-Hbb^d, and C57BL/6J-Hbb^{p}c^h and inbred strains WB/ReJ and 129/Sv were a gift from Dr Barry Whitney, Medical College of Georgia, Augusta.

Recombinant inbred mice The recombinant inbred strains used in these studies were derived from crossing mice of two unrelated but highly inbred progenitor strains, the offspring of which had been maintained independently under a regimen of strict inbreeding since the F_2 generation.[22] To qualify as recombinant inbred strains at least 20 generations of inbreeding after the F_2 generation were required. The seven recombinant inbred strains used here are called CXB (with the letters *D*, *E*, *G*, *H*, *I*, *J*, and *K* designating each strain) and were derived from a cross between the progenitor inbred strains BALB/cAnNBy (female) and C57BL/6JNBy (male).

Recombinant inbred strains were used to determine the number of genes controlling the hemolysis characteristic. This is possible because each recombinant inbred strain becomes fixed at each genetic locus for the allele of one of the progenitor strains. Therefore, a gradation of the characteristic of choice in the recombinant inbred strains (in comparison to the progenitor strains) is preliminary evidence for a polygenic mode of inheritance. Alternatively, if the characteristic of interest appears in the recombinant inbred strains in the same manner that it appears in one or the other of the progenitor strains, then a single gene mode of inheritance is indicated.[23,24]

Congenic inbred mice Congenic inbred strains are identical except for a short segment of chromosome containing the gene locus of interest. The congenic strains used here were produced by breeding and selecting for and transferring from one strain to another a short section of chromosome 7 that contained the loci for the Hb β chain.[25] These congenic strains, therefore, had RBCs that contained an Hb β chain type (ie, Hbb^d or Hbb^p) different from that normally present (Hbb^s) in that mouse strain, whereas the rest of the genome background of that mouse remained unchanged. This makes it possible to isolate the effect of the Hb β chain genes in the presence of a constant genomic background.[23]

RESULTS

RBC from inbred BALB/c mice formed malonyldialdehyde at a rate four times faster and showed more hemolysis in the presence of hydrogen peroxide than did red cells from C57BL/6 mice (Fig 1).

These hemolytic differences were confirmed by inspecting blood smears made after 60 minutes of incubation under conditions used in Fig 1. The smears of (hemolytic) BALB/c RBC showed cell fragments with only occasional intact cells, whereas the preparations from (nonhemolytic) C57BL/6 showed primarily intact cells (results not shown).

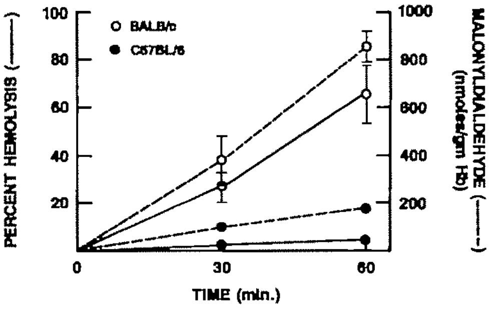

Fig 1. The percent hemolysis (solid line and left vertical axis) and malonyldialdehyde formation (broken line and right vertical axis) of RBC from inbred mice BALB/c (open circles) and C57BL/6 (closed circles). Vertical bars indicate ±1 SD (n = 4).

Table 1. Enzyme Activity

Mouse Strain	Glutathione Peroxidase
Progenitors	
1. BALB/c*	183 ± 47† (10)
2. C57BL/6	145 ± 43 (8)
Recombinant inbred mice	
3. CXB E	188 ± 20 (8)
4. CXB G*	149 ± 30 (13)
5. CXB H	219 ± 24 (8)
6 CXB I*	214 ± 24 (8)
7. CXB J	255 ± 25 (4)
8. CXB K	230 ± 21 (8)

The means were compared by using one-way analysis of variance ($P = .0001$). The variances were homogenous by Bartlett's test ($P > .05$). The differences among the eight means were analyzed by using the Student-Newman-Keul test. Sample no. 5 was significantly different from nos. 1, 4, 7, and 8; sample no. 6 was significantly different from nos. 4, 7, and 8; and sample nos. 1, 2, and 3 were significantly different from nos 4 and 8.

*Mice with RBC that hemolyze using methods described earlier.

†μmol NADPH/min/g Hb (mean ± 1 SD with the number of assays in parentheses).

Table 1 shows the glutathione peroxidase maximum velocity activities in red cells of BALB/c, C57BL/6, and their recombinant inbred offspring. There do not appear to be differences in enzyme activity that correlate with differences in hemolysis.

The effect of five antioxidant compounds on the hydrogen peroxide–induced hemolysis of BALB/c RBC is shown in Table 2. Butylated hydroxyanisole, butylated hydroxytoluene, and histidine each show a protective effect on the red cells at 3.0 mmol/L. Benzoate and mannitol (3.0 or 10.0 mmol/L) show little or no protection against hemolysis.

Figure 2A shows results obtained with RBC from 14 different inbred mouse strains that were either nonhemolytic (strains C57BL/10Sc, C57L, and STR) or hemolytic under hydrogen peroxide stress. Of the erythrocytes that hemolyzed, nearly all (91%) showed hemolysis of at least 30% to

Table 2. The Effect of Antioxidants on the Hydrogen Peroxide–Induced Hemolysis of RBCs From BALB/c Mice

Antioxidant	Hemolysis at 30-min Incubation
Control (no antioxidant)	100%*
Butylated hydroxyanisole (3.0 mmol/L)	19 ± 3% (4)
Butylated hydroxytoluene (3.0 mmol/L)	4 ± 5% (4)
Histidine	
10.0 mmol/L	18 ± 8% (3)
3.0 mmol/L	50 ± 17% (3)
Benzoate	
10.0 mmol/L	99 ± 12 (3)
3.0 mmol/L	89 ± 10 (3)
Mannitol	
10.0 mmol/L	96 ± 7 (3)
3.0 mmol/L	84 ± 12 (3)

*For purposes of comparison the control hemolysis is set to 100%. Values shown are means ± 1 SD. The values in parentheses are the numbers of separate assays done.

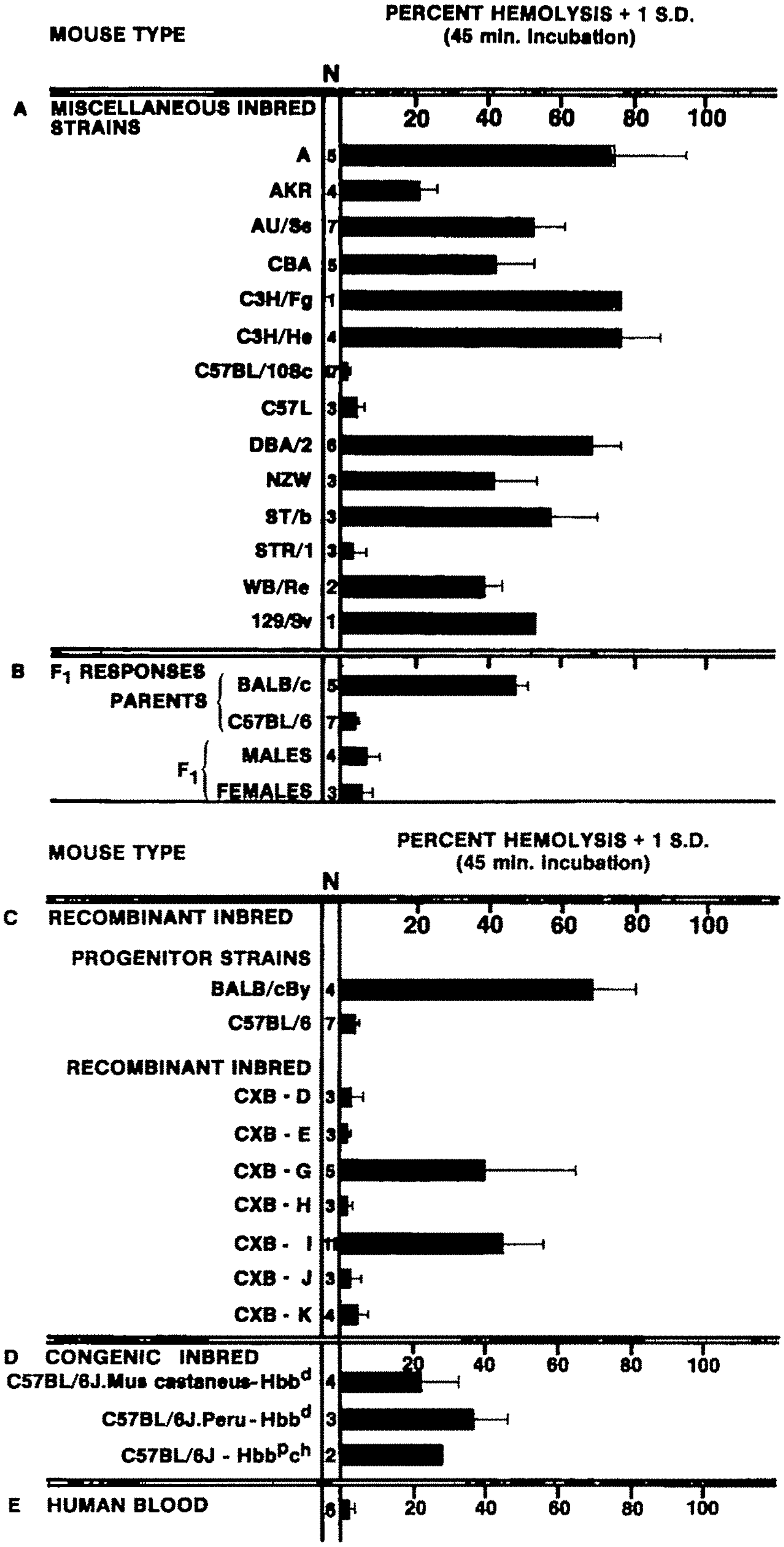

Fig 2. The mean percent hemolysis (±1 SD) of RBC from (A) miscellaneous inbred mice, (B) inbred mice BALB/c and C57BL/6 and their F_1 offspring, (C) recombinant inbred mice CXB, (D) congenic inbred mice, and (E) healthy adult humans.

40% during the 45-minute incubation. RBC from strain AKR were the only exception showing hemolysis slightly >20%. Those red cells that did not hemolyze showed hemolysis <10%. Therefore, a zone exists between 10% and 20% hemolysis that separates the red cells that hemolyze from those that do not.

To further characterize the genetic basis of this hemolysis/nonhemolysis trait we examined blood of mice from F_1 offspring, recombinant inbred, and congenic inbred strains.

The mating of BALB/c females (having had hemolytic red cells) with C57BL/6 males (having had nonhemolytic RBC) generated an F_1 population, all of which, male and female, showed no hemolysis, identical to the male parent (Fig 2B). These results indicated that the hemolytic trait is recessive to the nonhemolytic trait and that neither response is sex linked.

Seven recombinant inbred strains (see Methods and Materials) were available from a progenitor mating between BALB/c and C57BL/6 inbred mice. The red cells of mice from two recombinant inbred strains (CXB-G and CXB-I) showed oxidant-sensitive hemolysis similar to the BALB/c progenitor, and the remaining five showed low hemolysis levels equivalent to the C57BL/6 progenitor (Fig 2C).

The erythrocyte response of mice of three congenic inbred strains (see Methods and Materials) of C57BL/6 to hydrogen peroxide was investigated next (Fig 2D). Note that the Hb β chain gene (Hbb) in these congenic strains was of the (Hbb^d) or p-diffuse (Hbb^p) type, not the single type Hbb^s as seen in the C57BL/6. Note also that RBC from the congenic mice showed significant hemolysis in striking contrast to the nonhemolysis of the erythrocytes from the C57BL/6 strain alone (compare with C57BL/6 in Fig. 2C).

The nonhemolytic response of human RBCs to oxidant stress is shown in Fig 2E.

Table 3 summarizes and groups all of the inbred, recombinant inbred, and congenic inbred mouse red cell responses into either a hemolytic or nonhemolytic category. Table 3 also lists the types of Hb β chain carried by each inbred mouse RBC. Note that the single Hb β chain was present exclusively in the RBCs that did not hemolyze and the diffuse or p-diffuse Hb β chains were present only in the erythrocytes that hemolyzed.

DISCUSSION

These data document a novel genetic dimorphism in mice. The trait, hemolysis/nonhemolysis, is defined by whether RBC from inbred mice will hemolyze upon exposure to the oxidative stress of hydrogen peroxide. In addition, the hemolysis/nonhemolysis is consistent with the rate of membrane lipid oxidation (ie, malonyldialdehyde formation).[13,27] In other words, red cells that showed high hemolysis also showed a fast rate of malonyldialdehyde formation whereas red cells that showed little or no hemolysis demonstrated lower rates of malonyldialdehyde formation. The use of a well-known oxidizing agent like hydrogen peroxide, the increased formation of malonyldialdehyde, and the protection of cells from hemolysis by antioxidants support the concept that whether a mouse red cell type is hemolytic or nonhemolytic is somehow linked to differences in how each cell type responds to oxidant stress.

Table 3. Summary Table Showing Hbb Allotypes in Erythrocytes From Inbred Mice

Inbred Strains	Hbb Type
Nonhemolytic	
C57BL/6	s
C57BL/10Sc	s
C57L	s
STR/1	s
CXB-D	s
CXB-E	s
CXB-H	s
CXB-J	s
CXB-K	s
Hemolytic	
A	d
AKR	d
AU/Ss	p
BALB/c	d
BALB/cBy	d
CBA	d
C3H/He	d
C3H/Fg	d
DBA/3	d
NZW	d
ST/b	d
WB/Re	d
129/Sv	d
CXB-G	d
CXB-I	d
C57BL/6J.*Mus-castaneus-Hbb*^{d}	d
C57BL/6J.PERU-Hbb^d	d
C57BL/6J -Hbb^{p}c^h	p

Data from Staats.[28]

Why do RBC from some inbred mouse strains hemolyze so readily whereas others do not? As will be discussed, data from this part of the investigation indicated that hemolysis/nonhemolysis (*a*) exhibits characteristics of Mendelian inheritance, (*b*) cannot be immediately explained by postulating differences in glutathione peroxidase activity or levels of GSH in the red cells, and (*c*) shows a 1:1 association with the type of Hb β chain carried by the red cell.

A mating between a male inbred mouse with hemolytic red cells and a female inbred mouse with nonhemolytic red cells produced F_1 offspring with red cells that were all nonhemolytic. This indicates (*a*) the single-gene control of this trait, (*b*) that the hemolytic characteristic is recessive to the nonhemolytic characteristic, and (*c*) the autosomal chromosome (non–X-linked) location of the controlling gene. The single-gene control of this trait was further indicated by the results from mice of the recombinant inbred strains. In this case, offspring from a cross between progenitor mice (one with nonhemolytic and the other with hemolytic red cells) yielded two distinct groups, those with red cells that do hemolyze and those with red cells that do not. The nature of this trait (ie, discrete hemolysis or nonhemolysis, similar to the progenitor strains) again indicates a single controlling gene.[23,24]

This single controlling gene may control the Hb β chain (Hbb) synthesis as indicated by the 1:1 correlation between the trait and the type of Hb β chain contained with the red cell (Table 3). Specifically, nonhemolysis is correlated with the presence of the single Hb β chain gene (Hbb^s) and hemolysis is correlated with the p-diffuse (Hbb^p) or diffuse (Hbb^d) β chain genotypes.

The best evidence that the Hb β chain directly influenced the hemolysis trait came from the results with congenic inbred strains. RBC from inbred mice C57BL/6 normally carry the single Hb β chain associated with nonhemolysis. However, through interbreeding, crossbreeding, and selection, C57BL/6 mice with red cells carrying the diffuse or p-diffuse Hb were produced.[24] Therefore, the effects of diffuse or p-diffuse Hb β chains were evaluated against the genomic background of a mouse that normally carried the single Hb β chain. The fact that three separate C57BL/6 congenic inbred lines showed hemolysis was strong evidence that the presence of diffuse or p-diffuse Hb β chains is a necessary requirement in the sequence of events leading to the initiation of RBC hemolysis under the conditions used here.

Two points of caution should be made. The first regards the congenic inbred strain: other tightly linked genes may have been carried along with the Hbb^d and Hbb^p β chain genes as they were transferred into the C57BL/6 genome on the small section of chromosome.[24] Although this chromosomal segment is only about 2% of the total donor genome, it is possible that a gene linked to but distinct from Hbb is actually responsible for the hemolysis/nonhemolysis trait. Second, the RBCs from all mice strains did not hemolyze to the same extent. For example, inbred strains A, C3H/Fg, and DBA/2 showed a mean hemolysis of 70% to 80%. Strain AKR showed a mean hemolysis of only slightly more than 20%. The three congenic inbred strains showed hemolysis in the 20% to 40% range (Fig 3). This suggests that hemolysis is under the control of at least two sets of factors. One controls whether hemolysis will occur (i, hemolysis initiation factors), and a second set controls the extent of hemolysis once initiated. The Hb β chain type appears to function in the present system as a hemolysis initiation factor since it does not appear to correlate closely with the extent of hemolysis.

So far we have no evidence that red cell metabolism plays any role in these hemolysis differences. Indeed, the experimental protocol was developed specifically to exclude metabolic considerations. First, there was no glucose or other substrate in the wash or incubation buffers to fuel any of the energy-producing pathways of the red cell.[28] Second, red cells store only small amounts of glycogen,[29] eliminating glycogen as a source of metabolic energy in the washed cells. The cells in the present system, therefore, have only their pools of (nonrenewable) endogenous metabolites available to cope with the oxidative stress to which they are exposed. As the example that follows indicates, the size of these pools is not nearly adequate to significantly blunt the stress of the hydrogen peroxide. Third, sodium azide was included in the wash and incubation buffers to inhibit the endogenous catalase.[30] Fourth, glutathione peroxidase activities were measured (Table 1) and showed no consistent correlation with the tendency of the red cell type to hemolyze. And fifth, levels of GSH were measured in the washed cells just before testing for hemolysis; (hemolytic) BALB/c red cells contained 5.9 ± 0.3 μmol GSH/g Hb (mean ± SD) and (nonhemolytic) C57BL/6 cells contained 6.2 ± 0.7 μmol GSH/g Hb. These mean GSH values are within 5% of one another and agree favorably with GSH levels in human red cells (ie, 6.6 ± 1.0 μmol GSH/g Hb[17]). There is, therefore, 0.084 μmol of GSH in 1 mL of the washed cell suspension (calculation based on 14.0 mg Hb/mL). Even if all of this GSH were used to detoxify the 2.0 μmol/mL hydrogen peroxide (for example, by the action of glutathione peroxidase), only 4% of the oxidant would be affected, not enough to have any influence on hemolysis.

Given, on the one hand, the indication of a minimal role of cell metabolism (described earlier) and, on the other hand, the 1:1 correlation between the Hb β chain type and hemolysis (described previously, Table 3), it would appear most reasonable to explore the Hb type as a critical factor in these hemolytic differences.

Hb has been shown by others to play a major role in red cell response to oxidative stress. For example, Hb is necessary for lipid peroxidation to occur. In red cell membranes washed free of Hb, hydrogen peroxide does not cause lipid peroxidation unless Hb is added back.[10,11] The Hb somehow translates the oxidative potential of the hydrogen peroxide into potentials that oxidize the membrane lipids. The oxygen binding state of Hb is also important. Trotta et al,[31] using tBH as the oxidizing stress agent, show that methemoglobin slows lipid peroxidation, perhaps by absorbing the oxidative metabolites before they get to the lipids. Oxyhemoglobin supports lipid peroxidation.

In defining the role of Hb in the present system, our current working hypothesis centers on a specific interaction between Hb and the cell membrane. Human Hb has been shown to interact with high affinity with the red cell membrane band 3 protein through a cysteine residue at position 93 on the β chain.[32] Mouse Hb β chains also have a cysteine at position 93. In addition, however, each diffuse and p-diffuse Hb β chain (associated with hemolysis) has an additional cysteine residue at position 13. The single Hb β chain (associated with nonhemolysis) has a glycine residue at position 13.[33-35] Having the additional reactive sulfhydryl at position 13 may be the factor that makes the diffuse and p-diffuse Hb-containing red cells more susceptible to hemolysis during oxidative stress by providing an additional site for the Hb to react with the membrane. This and related hypotheses are under investigation.

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Cloning and Nucleotide Sequence of a Mouse Erythrocyte β-Spectrin cDNA

By Livia Cioè, Pekka Laurila, Pacifico Meo, Keith Krebs, Steven Goodman, and Peter J. Curtis

A rabbit monospecific antibody for mouse β-spectrin was used to screen a mouse anemic spleen cDNA expression library. A mouse β-spectrin cDNA clone was isolated and identified by its ability to make mouse β-spectrin-like antigens in *Escherichia coli*. This clone was used to probe total RNA from various mouse tissues. Anemic spleen RNA showed two strongly hybridizing RNA species of approximately 6 and 8 kb. Two very faintly hybridizing bands of about 6 kb and 10 kb could also be seen in total mouse brain RNA. All of these bands could be detected after hybridization under both stringent and nonstringent conditions. This suggests that erythroid β-spectrin may also be expressed in the brain. No bands could be detected in kidney, liver, or spleen RNA. Southern blot analysis of mouse genomic DNA showed a single hybridizing band after digestion with several restriction endonucleases even under nonstringent conditions. Nucleotide sequencing of the cDNA insert revealed almost complete identity between the N-terminus of the deduced amino acid sequence of the cDNA clone and the C-terminal 15 amino acids of a peptide derived from the β-8 repeat unit of human erythrocyte β-spectrin. The deduced amino acid sequence contained most of the conserved amino acids characteristic of the 106 amino acid repeat unit first found in human α-spectrin and thus provides the first evidence for a complete 106 amino acid repeat unit structure in β-spectrin.

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SPECTRIN IS THE predominant component of the erythrocyte membrane skeleton, which forms a two dimensional protein lattice on the cytoplasmic side of the membrane. Spectrin consists of two polypeptide chains, α- and β-subunits (M_r approximately 240,000 and 220,000, respectively), which associate as heterodimers and tetramers to form a rod-like structure approximately 100 nm long. The hetero-oligomers in turn interact through the β subunit with ankyrin and at one end with band 4.1 and short actin filaments to produce a submembranous network.[1] The membrane cytoskeleton confers on the erythrocyte its shape and elasticity, and defects in this structural network are considered to be the cause of the fragility of red cells in various forms of inherited hemolytic anemias; these include hereditary spherocytosis (HS), elliptocytosis (HE), and pyropoikilocytosis (HPP).[2,3] Detailed analysis of the cytoskeletal components in cases of HPP and HE has revealed a deficiency in the capacity of spectrin dimers to form tetramers. This deficiency correlates with an increased sensitivity of the α-spectrin subunit to limited proteolytic digestion,[4,5] implying that this subunit is the site of the defect. In most cases of HS, a deficiency of spectrin correlates with the severity of the disease, although the reason for the spectrin deficiency is not known.[6] A deficiency of β-spectrin has also been noted in one case of HE.[7] Analogous hemolytic anemias have been identified in the mouse, where deficiencies of α-spectrin (*sph/sph*), β-spectrin (*ja/ja*), and ankyrin (*ab/nb*) were observed.[8]

Considerable progress has been made in determining the amino acid sequence of human erythrocyte α- and β-spectrin, from which it was noted that the spectrin subunits contain a 106 amino acid repeat unit.[9,10] The recent isolation and characterization of α-spectrin cDNA and genomic clones will facilitate the completion of the amino acid sequence analysis for α-spectrin.[11-14] In this report we describe the isolation of a mouse erythrocyte β-spectrin cDNA by immunological screening of a mouse anemic spleen expression cDNA library. This probe was used to study the expression of β-spectrin-related mRNA species in various tissues as well as to determine the number of related genes in the mouse genome. The nucleotide sequence of the cloned cDNA provides the first evidence of a complete 106 amino acid repeat unit in β-spectrin, and this result supports the model that the 106 amino acid repeat unit is the basic structure of both spectrin subunits.

MATERIALS AND METHODS

Antibodies. Antiserum to total mouse spectrin was kindly supplied by P.A. Marks (Sloan-Kettering Institute, New York).

The serum against the β subunit of mouse spectrin was prepared as follows: Erythrocyte ghosts were prepared from 70 mL freshly drawn mouse (male C-57 black) blood as described for human erythrocyte ghosts, and spectrin heterodimers were purified to homogeneity as previously described.[15,16] Twenty milliliters of the heterodimer solution (20 mmol/L Tris, pH 7.6, 1 mmol/L EDTA) was added to 20 mL of a solution of 6 mol/L urea, 300 mmol/L NaCl, 20 mmol/L Tris, pH 7.6, 1 mmol/L EDTA, 1 mmol/L dithiothreitol (DTT) to give a final concentration of 3 mol/L urea, 150 mmol/L NaCl, 20 mmol/L Tris, pH 7.6, 1 mmol/L EDTA, 0.5 mmol/L DTT. The heterodimer solution was stirred overnight at 4°C to dissociate the spectrin subunits and subsequently was loaded onto a DE-52 anion exchange column (1 cm × 10 cm) that had been equilibrated with the same buffer. The β-spectrin subunit was eluted from the column with 50 mL of column buffer, and the purity was evaluated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

A male New Zealand white rabbit was injected with 80 µg of the pure β-spectrin in an equal volume of Freund's complete adjuvant.

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followed by a booster injection of 40 μg in incomplete Freund's adjuvant after 4 weeks and an identical second booster injection 6 weeks after the initial injection. Blood was drawn 1 week after the second booster, and the serum collected. The IgG fraction was obtained by precipitation with 35% $(NH_4)_2SO_4$, and the pellet resuspended in 5 mmol/L Na_2HPO_4/NaH_2PO_4, 150 mmol/L NaCl, 0.05% Tween 20, 1 mmol/L Na azide, pH 7.4, followed by dialysis against the same buffer. To eliminate antibody that cross-reacted with brain spectrin, the IgG fraction was passed over a sepharose 4B column to which mouse brain spectrin (240/235) had been covalently bound, and the noncross-reacting antibody that eluted from the column was collected. The specificity of the antibody was determined by Western blot analysis and found to react exclusively with the erythrocyte β-spectrin subunit (see Results section). Cross-reactivity of the antibody with erythrocyte α-spectrin and mouse brain 240/235 spectrin was undetectable at a 1:100 dilution.

Screening of the expression library of mouse anemic spleen. The construction of expression libraries in the plasmid vectors pUC 7, pUC 8, and pUC 9 has been described.[17] The cDNA libraries were immunologically screened using the procedures described by Helfman et al,[18] and under containment in accord with NIH guidelines.

Immunoautoradiography. Escherichia coli polypeptides were prepared by sonication in the presence of Triton X-100 and lysozyme[17] and were estimated by the procedure of Schaffner and Weissman.[19] Mouse erythrocyte ghosts were prepared by the methods of Fairbanks et al.[20]

Proteins were separated by SDS/6/12% electrophoresis based on the system of Laemmli[21] and Fairbanks et al.[20] Immunoblots and autoradiography were performed as described.[22] Alternatively, immunoblots were developed using biotinylated anti-rabbit $F(ab')_2$ IgG and streptavidin-peroxidase complex (supplied by Zymed Inc, San Francisco) according to French and Maul.[23]

Isolation and electrophoresis of RNA. Total and poly(A^+) RNA were prepared from spleens of anemic mice as previously described.[24] Poly(A^+) RNA was fractionated on a 1.2% agarose gel in the presence of 2.2 mol/L formaldehyde.[25] Transfer to nitrocellulose and prehybridization and hybridization procedures were essentially as previously described.[24,26] Calf thymus primers and *E coli* DNA polymerase I were used to label the cloned sequences.[27]

Isolation of mouse nuclear DNA. Total mouse nuclear DNA was isolated as described previously.[28] The DNA was digested with restriction endonucleases, and the fragments were fractionated on a 0.8% agarose gel. Transfer to nitrocellulose filters and prehybridization and hybridization procedures were essentially as described previously.[28]

DNA sequence analysis. The cDNA insert of pβ58 was purified from a low melting agarose gel, ligated to M13 mp 18/19 RF DNA, and transfected into JM103. Sequential deletions of the insert were produced by exonuclease III digestion of linearised M13 RF.[29] Single strand phage DNA was prepared from colorless plaques, and sequences were determined by using the dideoxynucleotide chain-termination method.[30,31]

RESULTS

Immunological screening of the cDNA library. Twenty-thousand transformants for each pool of the expression library (60,000 in total) were plated onto agar plates containing ampicillin to give ≈1,000 colonies per plate. Colonies were replicated onto nitrocellulose filters and immediately lysed.[29] The filters were then incubated at room temperature with the rabbit antiserum to the β chain of mouse spectrin diluted 1:100. One positive colony was obtained from such a screening. On replating the colony onto ampicillin-containing agar plates and rescreening with the same antiserum, almost 50% of the plated colonies showed strong reactivity with the antiserum. From six of these subcloned colonies plasmids were prepared and analysed on 1% agarose gel after digestion with the restriction enzyme Pst1. Each contained ≈750 base pair (bp) inserts which cross-hybridized. Further analyses have been carried out with one of the six colonies containing the plasmid pβ58.

Immunological characterization of pβ58 peptides. A bacterial lysate was prepared from an overnight culture of *E coli* DH 1-pβ58, electrophoresed on a SDS/12% polyacrylamide gel, and transferred to a nitrocellulose filter. Fig 1 shows the immunoautoradiography using the anti-β spectrin serum on that lysate. One major polypeptide (MW 25,000) is produced by *E coli* DH-1 transformed by pβ58 (Fig 1B). On the other hand, the same antiserum detected no specific polypeptides on an immunoblot (Fig 1A) of the lysate from *E coli* DH-1, transformed by pMαSp1 that carries sequences of the mRNA for mouse α-spectrin.[17] However, two faint bands of low molecular weight may be seen on the immunoblot, but these most probably represent nonspecific binding by the antibodies to the immunoblot.

To demonstrate that the plasmid pβ58 carries a cDNA insert that contains sequences of the mouse β-spectrin mRNA, *E coli* DHI containing pβ58 was grown overnight at 37°C. A lysate was prepared from the above culture and

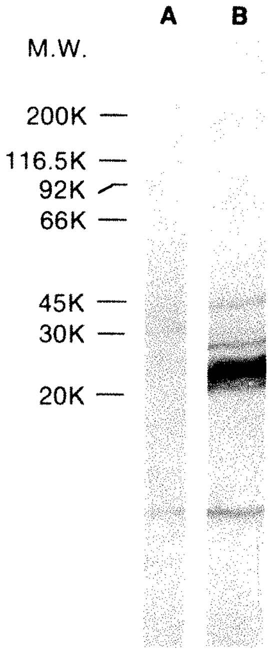

Fig 1. Detection of mouse β-spectrin-like antigens in *E coli* (strain DH1) carrying (A) pMαSp1 mouse α-spectrin cDNA [17]. (B) pβ58. Cells were lysed by sonication in the presence of Triton X100, DNase, and lysozyme, and the lysates were electrophoresed in a SDS/12% polyacrylamide gel. The proteins were transferred to nitrocellulose and reacted with rabbit anti-mouse β-spectrin IgG at a dilution of 1:100, followed by ^{125}I-labeled $F(ab')_2$ fragment to rabbit IgG. Each lane contained approximately 10 μg of total bacterial proteins.

electrophoresed in a SDS/12% polyacrylamide preparative gel 1.5 mm thick. Gel slices containing the 25 kDa polypeptide detected by the antiserum were excised, homogenized, and injected into rabbits. Sera from rabbits immunized with the 25 kDA protein derived from pβ58 were tested subsequently for their ability to react with spectrin. The immunoblots (Fig 2) show erythrocyte ghost proteins that reacted with α-spectrin antibodies[17] (Fig 2A), DH1-pβ58 antiserum (Fig 2B), β-spectrin antiserum (Fig 2C), and ankyrin antiserum (Fig 2D). The sera from the rabbits immunized with the MW 25,000-β58 peptide reacted specifically with the β-subunit of mouse spectrin, which was clearly resolved from the α-subunit (Fig 2B). This result indicates that the MW 25,000-β58 protein contains an antigenic determinant of the mouse β-spectrin molecule.

Size of the encoded mRNA and expression in different tissues. Total RNA from mouse brain, kidney, liver, normal, and anemic spleen was electrophoresed on a 1% agarose gel containing 2.2 mol/L formaldehyde and then transferred to nitrocellulose filters. The blots were hybridized with the 720 bp β-spectrin insert from pβ58 (Fig 3). The insert from pβ58 hybridized with two RNA species in mouse anemic spleen RNA, one about 8 kb and a faster one of about 6 kb (Fig 3), whereas previously we had shown that the α-spectrin insert detected only a single band of about 8 kb. The relative intensities of the two β-spectrin bands were unaffected by the stringency of the hybridization conditions. RNA from mouse brain also showed two faint bands after hybridization with the pβ58 insert. The lower molecular weight brain RNA band comigrated with the 6 kb band from mouse anemic spleen, whereas the upper band showed slower mobility (about 10 kb) than the 8-kb band detected in anemic spleen RNA (Fig 3). The signals in the brain RNA were detectable only after exposure for five days. No hybridization was detected in mouse kidney, liver, or normal spleen after hybridization with the β-spectrin insert under both stringent and nonstringent conditions.

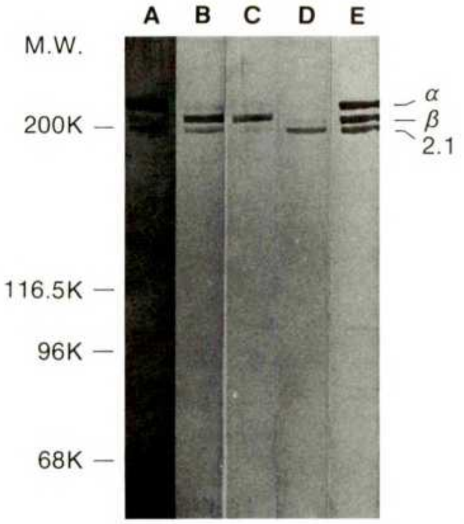

Fig 2. **Reactivity of the antiserum against bacterial peptides of *E coli* (pβ58). Samples (~10 μg) of mouse erythrocyte membrane proteins were electrophoresed in a SDS/6% polyacrylamide gel. After electrophoretic transfer to nitrocellulose, the blots were allowed to react with the following: (A): a 1:50 dilution of rabbit serum raised against pMαSp1-directed protein [17]; (B): a 1:100 dilution of rabbit serum immunized with pβ58 directed protein; (C): a 1:100 dilution of antiserum to mouse β-spectrin; (D): a 1:1000 dilution of antiserum to mouse ankyrin; (E): a 1:1000 dilution of antiserum to mouse erythrocyte membrane proteins. After one hour incubation the filters were incubated with biotinylated anti-rabbit F(ab')$_2$ IgG and streptavidin peroxidase complex.**

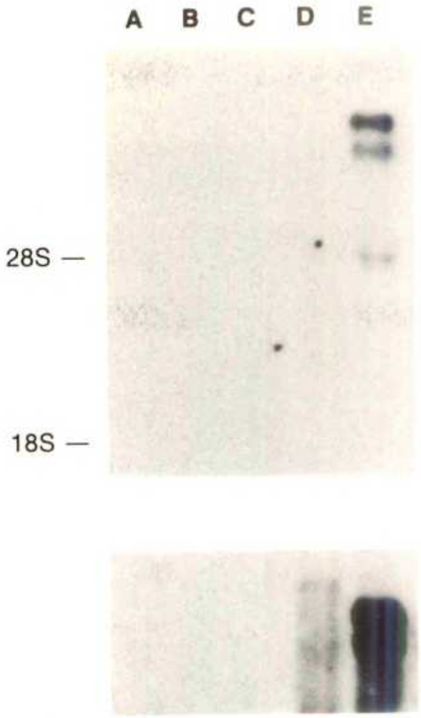

Fig 3. **Size of pβ58 encoded mRNA. Total cellular RNA (25 μg) from different mouse tissues was electrophoresed in a 1% agarose gel containing 2.2 M formaldehyde and transferred to nitrocellulose. The filter was hybridized with ^{32}P-labeled pβ58. (A) kidney, (B) liver, (C) normal spleen, (D) brain, (E) anemic spleen. Ribosomal RNA was used as a size marker. The insert below is the result of longer exposure of the filter to visualize the faint bands seen in brain RNA.**

Nucleotide sequence of mouse β-spectrin cDNA. The nucleotide sequence was determined by subcloning the cDNA insert into M13mp 18/19 from which deletions were derived using exonuclease III (Fig 4a).[29] The insert contains a dC-dG tail only at one end. Examination of the reading frames showed only one that is open for its entire length. The deduced amino acid sequence shows homology with the C-terminus of a peptide assigned to the repeat unit 8 of human β-spectrin,[10] where 13 of 15 amino acids are identical with only one mismatch, one amino acid being unassigned in the peptide sequence (Fig 4B). Presumably, the deduced amino acid sequence represents the β8-10 repeat units of mouse erythrocyte β-spectrin. In the β8 repeat unit, the mouse sequence retains seven of ten of the conserved amino acids, including tryptophan at position 45, while in the β9 repeat unit, nine of 15 are conserved but tryptophan is not found at position 45, and in the β10 repeat unit, seven of 11 are conserved, including tryptophan at position 45. One

A

```
                                                            60                                                          120
CTGCAGGCTTTCCTGCAAGATCTGGATGAC TTCAAGGCCTGGCTGTCCATGGCCCAAAAG GCAGTGGCCTCCGAGGACATGCCTGAGTCA CTCCCAGAGGCGGAGCAGCTCCTACAGCAG
L  Q  A  F  L  Q  D  L  D  D   F  K  A  W  L  S  M  A  Q  K   A  V  A  S  E  D  M  P  E  S   L  P  E  A  E  Q  L  L  Q  Q

                                                           180                                                          240
CATGCAGCCATTAAGGAAGAGATTGATGCG CACAGAGACGATTACCATAGGGTCAAGGCC TCTGGGGAGAAAGTGATCGAAGGCCAGACC GACCCAGATTACCAGCTTCTGGGCCAGCGG
H  A  A  I  K  E  E  I  D  A   H  R  D  D  Y  H  R  V  K  A   S  G  E  K  V  I  E  G  Q  T   D  P  D  Y  Q  L  L  G  Q  R

                                                           300                                                          360
TTAGAGGGCCTAGATACTGACTGGGATGCT CTACGCCGGATGTGGGAGAGCCGAGGGAAC ACGCTCACCCAGTGCCTGGGCTTTCAGGAG TTCCAGAAAGATGCCAAGCAGGCTGAAGCC
L  E  G  L  D  T  D  W  D  A   L  R  R  M  W  E  S  R  G  N   T  L  T  Q  C  L  G  F  Q  E   F  Q  K  D  A  K  Q  A  E  A

                                                           420                                                          480
ATCCTCAGCAACCAGGAATACACTCTGGCT CACTTGGAGCCTCCGGACTCCCTAGCGGCA GCAGAGGCGGGCATCCGAAAGTTTGAGGAT TTCCTAGTATCTATGGAAAACAACCGAGAC
I  L  S  N  Q  E  Y  T  L  A   H  L  E  P  P  D  S  L  A  A   A  E  A  G  I  R  K  F  E  D   F  L  V  S  M  E  N  N  R  D

                                                           540                                                          600
AAGATCCTGAGTCCTGTGGACTCTGGCAAC AAGCTGGTGGCCGAGGGCAACCTGTATTCG AACAAGATCATGGAGAAGGTGCAGCTGATT GAAGACAGGCACAAGAAGAACAATGAGAAG
K  I  L  S  P  V  D  S  G  N   K  L  V  A  E  G  N  L  Y  S   N  K  I  M  E  K  V  Q  L  I   E  D  R  H  K  K  N  N  E  K

                                                           660
GCCCAGGAGGCTACAGTTCTCCTAAAAGAC AACCTGGAGTTGCAGAACTTCCTCCAGAAC TGCAAGGAGCTCACTCTCTGGATCAATGAC AAGCTGCTGACGTCTCCAGA
A  Q  E  A  T  V  L  L  K  D   N  L  E  L  Q  N  F  L  Q  N   C  K  E  L  T  L  W  I  N  D   K  L  L  T  S  P  D
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B

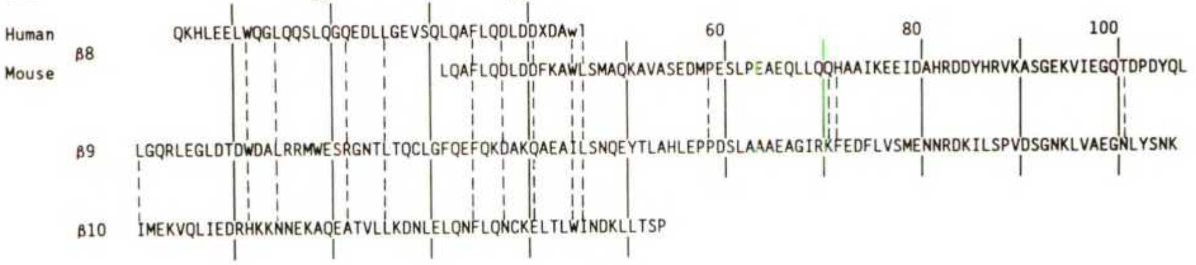
20
40
60
80
100
Human
β8
Mouse
QKHLEELWQGLQQSLQGQEDLLGEVSQLQAFLQDLDDXDAw1
LQAFLQDLDDFKAWLSMAQKAVASEDMPESLPEAEQLLQQHAAIKEEIDAHRDDYHRVKASGEKVIEGQTDPDYQL
β9
LGQRLEGLDTDWDALRRMWESRGNTLTQCLGFQEFQKDAKQAEAILSNQEYTLAHLEPPDSLAAAEAGIRKFEDFLVSMENNRDKILSPVDSGNKLVAEGNLYSNK
β10
IMEKVQLIEDRHKKNNEKAQEATVLLKDNLELQNFLQNCKELTLWINDKLLTSP

Fig 4. **(A) Nucleotide and deduced amino acid sequence of the mouse β-spectrin (pβ58) cDNA clone. (B) Deduced amino acid sequence of mouse β-spectrin aligned as 106 amino acid repeat units. Above the mouse amino acid sequence is shown the sequence of a peptide from human β-spectrin and assigned to the β8 repeat unit [10]. The dotted lines indicate the position of conserved amino acids as follows: 1-I, 12-W, 15-L, 22-R, 26-L, 35-F, 38-D, 41-D/E, 45-W, 46-I, 48-D/E, 59-G, 71-K/R, 72-H, 101-H.**

anomaly was noted; an extra amino acid must be placed between the β8 and β9 repeat unit to maintain optimal alignment of the conserved amino acids. For the human α- and β-spectrin amino acid sequences so far, the 106 amino acid repeat unit is invariant. Nucleotide sequencing of overlapping cDNA clones will determine if the extra amino acid has arisen from the cloning procedure, an aberrant mRNA, or if it is a property of the functional mRNA.

Probing of mouse genomic DNA with the β-spectrin insert. Nuclear DNA isolated from mouse liver was digested with restriction endonucleases *Bam*HI, *Eco*RI, *Hind*III, and *Pst*I and run on a 0.8% agarose gel and transferred to nitrocellulose. The blots were hybridized with the ^{32}P-labeled 720 bp insert from pβ58 (Fig 5). The insert hybridized with only a single band of various sizes in the *Bam*HI, *Eco*RI, and in the *Hind*III digests. Only the PstI digest gave two bands (Fig 5). The stringency of the hybridization conditions had no effect on the number of bands detected. These results indicate only a single gene for mouse erythrocyte β-spectrin.

DISCUSSION

Indentification of the mouse erythrocyte β-spectrin cDNA has relied on an initial screening with a monospecific antibody against mouse erythrocyte β-spectrin. Subsequently, we demonstrated that a polypeptide produced in *E coli* transfected with the β-spectrin cDNA clone was capable of eliciting antibodies with specificity for mouse erythrocyte β-spectrin. The identity of the clone was confirmed by nucleotide sequencing. The N-terminus of the deduced amino acid sequence matched, with only one mismatch, the

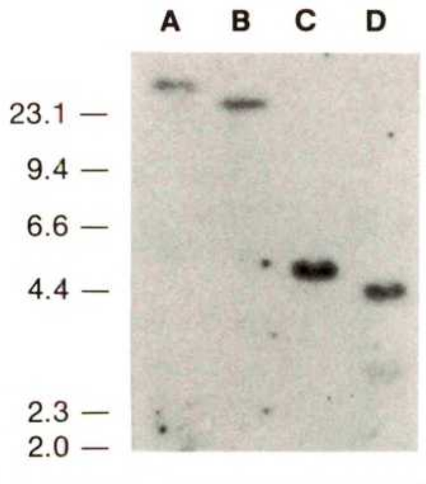

Fig 5. **Probing of mouse genomic DNA by the pβ58 insert. Mouse DNA digested with restriction enzymes was electrophoresed in 0.8% agarose, transferred to nitrocellulose, and hybridized with ^{32}P-labeled pβ58. (A) *Bam*HI, (B) *Eco*RI, (C) *Hind*III, (D) *Pst*I.**

amino acid sequence of a peptide from the β8 repeat unit of human erythrocyte β-spectrin.[10] No other close homology was detected between the deduced sequence of the β-spectrin cDNA clone and known sequences of human erythrocyte β-spectrin. This was expected, since the sequence of our cDNA extends only into the β10 unit, while the next known peptide sequence is derived from the β11 repeat unit. The amino acid sequence for the mouse β8, β9, and β10 repeat units contains most of the highly conserved amino acids characteristic of the 106 amino acid repeat unit of human α- and β-spectrin except for the segment from approximately residue 40 of β9 to residue 30 of β10; in particular the tryptophans at positions 45 and 12 are missing. This segment of the β9 and β10 repeat units is presumably located opposite the anomalous α10 repeat unit of human α-spectrin[10] and thus may have a different secondary structure from the other β-spectrin repeat units to accommodate the α10 repeat unit.

Two RNA species for erythyroid β-spectrin were detected in mouse anemic spleen. These bands probably do not represent two different β-spectrin sequences, since both the bands could be detected under both stringent and nonstringent conditions. Furthermore, only one band could be detected with the β-spectrin probe in mouse genomic DNA after digestion with several restriction enzymes and hybridization under nonstringent conditions.[28] This band has been unequivocally assigned to chromosome 12 using somatic cell hybrids.[28] Therefore, the two β-spectrin RNA species detected in mouse anemic spleen might represent the same mRNA with different lengths of 3′ noncoding sequences, a situation that has been previously observed with other mRNAs.[32] However, it seems unlikely that they are derived from different splicing events, since only one β-spectrin polypeptide has been observed in mouse erythrocytes, unless splicing occurs in the 5′ or 3′ untranslated regions of the mRNA.

Our results showing that no different bands were detected under low- and high-stringency hybridizations also suggest that the erythroid spectrin genes must differ considerably from their nonerythroid counterparts at the nucleotide level. The low homology between the erythroid and nonerythroid β-spectrin molecules is further supported by our results showing that in other organs, ie, mouse liver, kidney, or normal spleen, no erythroid β-spectrin messages could be detected. However, in the brain two very faint bands were detected with our erythroid β-spectrin insert. The larger of these two brain mRNA species showed somewhat slower mobility than the larger band in anemic spleen. These messages could well represent the expression of erythroid β-spectrin-like sequences in the brain in addition to the nonerythroid spectrin or fodrin messages. The expression of such erythroid spectrin molecules in the brain has previously been suggested by antibody studies.[33] The difference in size of the larger brain mRNA species as compared with that seen in the anemic spleen could possibly be explained by differential splicing occurring in these different tissues. Thus, the erythroid β-spectrin mRNA is mainly expressed in hematopoietic tissues such as anemic spleen, although it can also be expressed in the brain, where it may play a role in the spatial differentiation of the nerve cell.

The erythrocyte β-spectrin plays a crucial role in the integrity of the cytoskeletal network. It has binding sites for α-spectrin, ankyrin, and band 4.1,[34,35] and it seems to be the limiting component for the formation of heterodimers and heterotetramers.[36] In the present study we have described the first nucleotide sequence for β-spectrin. The deduced amino acid sequence from our cDNA clone delineates a complete 106 amino acid repeat unit structure for β-spectrin, which had first been identified in α-spectrin. The mouse β-spectrin cDNA will be invaluable for the characterization of the structure of β-spectrin and its role in the genectic defects of hemolytic anemias in both man and mouse known to involve the β-spectrin subunit.

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A Unique 7p/12q Chromosomal Abnormality Associated With Recurrent Abortion and Hypofibrinogenemia

By Craig S. Kitchens, Amelia C. Cruz, and Jeffrey A. Kant

Recurrent first trimester abortions led to evaluation of a 25-year-old woman. Studies revealed she had hypofibrinogenemia (68 mg/dL) without evidence of dysfibrinogenemia or increased fibrinogen turnover. She was also found to have a unique 46, XX, t(7;12) (p 15.2;q 24.31) karyotype. Hypofibrinogenemia and identical chromosomal abnormalities were found in other members of her kindred. Southern blots of genomic DNA from the patient, her mother, and her daughter hybridized to human fibrinogen probes showed α, β, and γ fibrinogen genes to be present and without structural alterations when compared to normal controls. We conclude that the chromosomal abnormality and the hypofibrinogenemia are related but in an unclear manner. Because fibrinogen infusion in the proposita was associated with successful gestation, we also concluded that the chromosomal abnormality itself was not responsible for the repeated abortions but that fibrinogen concentration may be critical in securing implantation.

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THE MECHANISMS by which the fertilized ovum invades the uterine wall during implantation and the subsequent repair of invaded tissue are subjects of active investigation.[1-5] Presumably key in these processes are several proteases and other proteins[6,7] regulating the activity of proteases. No doubt other processes are operative, including some yet to be identified. Coagulation factors appear to play a role in implantation. Clinical derangements of fibrin homeostasis that result in unsuccessful pregnancy[9-14] suggest that fibrinogen and fibrin aid in securing the ovum and the morula in place following uterine invasion.[8]

In this report, we describe a woman with congenital hypofibrinogenemia, recurrent spontaneous abortion, and an unusual chromosomal abnormality. During one pregnancy, plasma fibrinogen levels were maintained by cryoprecipitate administration. That pregnancy was successful, whereas previous and subsequent pregnancies without cryoprecipitate resulted in spontaneous abortion. Hypofibrinogenemia and the unique chromosomal abnormality were shared by other members of her kindred, findings which suggest an association among the hypofibrinogenemia, fetal wastage, and the chromosomal abnormalities.

MATERIALS AND METHODS

All coagulation studies were performed using platelet-poor plasma prepared from whole blood, which was anticoagulated with 1/10th volume 3.8% sodium citrate. Coagulation studies and determination of antithrombin III, plasminogen, and fibrinogen degradation products were done as previously described.[15] Plasma fibrinogen concentration was determined by three methods: chronometric,[16] gravimetric,[17] and immunoreactive (Cal Biochem, San Diego).

Fibrinogen was isolated from plasma, reduced with 2-mercaptoethanol, and electrophoresed on SDS-PAGE (7.5% acrylamide for the separating gel and 4.0% for the stacking gel) by the Laemmli method.[18] Cytogenetic analyses were performed using peripheral blood lymphocytes grown by a microculture technique.[19] Geimsa banding was done by a modification of the technique of Seabright.[20]

Fibrinogen gene structure was determined in several subjects. EDTA-anticoagulated blood (10 to 50 mL) was used for preparation of leukocyte genomic DNA by lysis and washing of cells in JG buffer (0.25 mol/L sucrose, 0.005 mol/L $MgCl_2$, 0.025 mol/L KCl, 0.05 mol/L Tris-HCl, pH 7.4) containing 0.25% Triton X-100. Nuclei isolated in this fashion were washed in JG buffer and disrupted in 20 mmol/L EDTA, 1% SDS, and then digested overnight with 100 to 500 ug/mL proteinase K. After phenol/$CHCl_3$ extraction, 10 ug of genomic DNA was digested for 2 to 3 hours using two additions of restriction endonuclease (3 U enzyme/μg DNA). The digests were fractionated by size on a 0.7% agarose gel at 2 volts/cm overnight, denatured, neutralized, and the contents transferred to BA85 nitrocellulose paper essentially as described by Southern.[21] This blot was hybridized to 5 to 20 × 10^6 cpm of ^{32}P-nick translated[22] human α, β, or γ fibrinogen cDNAs[23] at 65 C. in 6 × SSC (1 × SSC) = 0.15 mol/L NaCl, 0.015 mol/L Na citrate) for 18 hours, then washed at 65 C. successively in 6 × SSC-0.1% SDS, 1 × SSC-0.1% SDS, and briefly in 0.1 × SSC-0.1% SDS. The cDNAs used contained coding information from the carboxy terminus extending 5′ to residue 154, 241, and 255 of the α, β, and γ chains, respectively. The blots were exposed to Kodak XAR-5 x-ray film at −70°C.

CASE REPORT AND SPECIAL STUDIES

A 25-year-old woman consulted us following two spontaneous first-trimester abortions. She now wished to become pregnant again. No records were available concerning the first abortion, which took place in her 12th week of gestation. Her plasma fibrinogen level early in the second pregnancy was 59 mg/dL. She experienced vaginal spotting in the 4th week and spontaneously aborted in the 8th week. No transfusions were administered. Several weeks later her fibrinogen level was 51 mg/dL. Neither she nor other family members reported other hemorrhagic events. When she consulted us, she was in normal health and not on contraceptives. Multiple

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Table 1. Coagulation Studies on Proposita

Test	Normal Range	Patient
Prothrombin time (s)	9.5-14.5	13.9
Partial thromboplastin time (s)	25-40	30
Thrombin time (s)	17-33	60
Fibrin degradation products (titre)	≤1:4	1:2
Fibrinogen, chronometric (mg/dL)	150-400	68
Fibrinogen, gravimetric (mg/dL)	150-400	76
Fibrinogen, immunoreactive (mg/dL)	150-400	80
Factor II activity (% normal)	70-150	100
Factor V activity (% normal)	70-150	88
Factor VII activity (% normal)	70-150	94
Factor VIII activity (% normal)	60-200	120
Factor VIII antigen (% normal)	60-200	115
Factor IX activity (% normal)	70-150	100
Factor X activity (% normal)	70-150	82
Factor XI activity (% normal)	70-150	95
Factor XII activity (% normal)	70-150	120
Factor XIII screen, 5 mol/L urea solubility	negative	negative
Plasminogen CTA/mL	2.3-3.8	2.8
Antithrombin III (% normal)	80-120	94

coagulation studies were performed (Table 1). Hypofibrinogenemia was repeatedly confirmed at times when levels of serum fibrinogen degradation products were normal.

Because of the possibility that her previous abortions had been the result of chromosomal abnormalities, chromosomal analysis was done, which revealed a karyotype of 46, XX, t(7;12) (p 15.2;q 24.31) (Figs 1 and 2).

Pedigree analysis was carried out on all available members (Fig 3). Note the correlation of the 7p/12q translocation and hypofibrinogenemia. The proposita's mother had not experienced spontaneous abortion, although her plasma fibrinogen level (140 mg/dL) was consistently higher than that of her daughter (68 mg/dL).

We wished to see if the 7p/12q or other chromosomal aberrations were generalized in hypofibrinogenemia and related disorders. We collected blood and successfully karyotyped three patients with hypofibrinogenemia and one patient each with afibrinogenemia and dysfibrinogenemia. Karyotypic analyses of these five patients were normal. Whereas these findings do not exclude the possibility that other fibrinogen abnormalities are associated with other translocations, it appears that 7p/12q abnormalities are not generalized in disorders of fibrinogen.

Fibrinogen isolated from the proposita's plasma was reduced with 2-mercaptoethanol into α, β, and γ chains. SDS-PAGE electrophoresis was performed using reduced normal fibrinogen as a control. No differences in mobility of this patient's fibrinogen chains were observed (Fig 4). Fibrinogen isolated from the proposita's plasma and normal plasma showed identical reptilase and thrombin clotting times at several different concentrations. The normal half-life of infused fibrinogen (see below) and absence of increased serum fibrinogen degradation products in this patient served as evidence against increased fibrinogen turnover, thus supporting the probability of decreased fibrinogen production. The nearly identical results of plasma fibrinogen concentrations using three quantitative methods and the normal electrophoresis and normal thrombin clotting times support but do not prove that the fibrinogen abnormality in this kindred represents hypofibrinogenemia rather than dysfibrinogenemia.

The hypofibrinogenemia associated with this syndrome prompted us to examine the structure of these subjects' fibrinogen genes. Genomic DNA was isolated from periph-

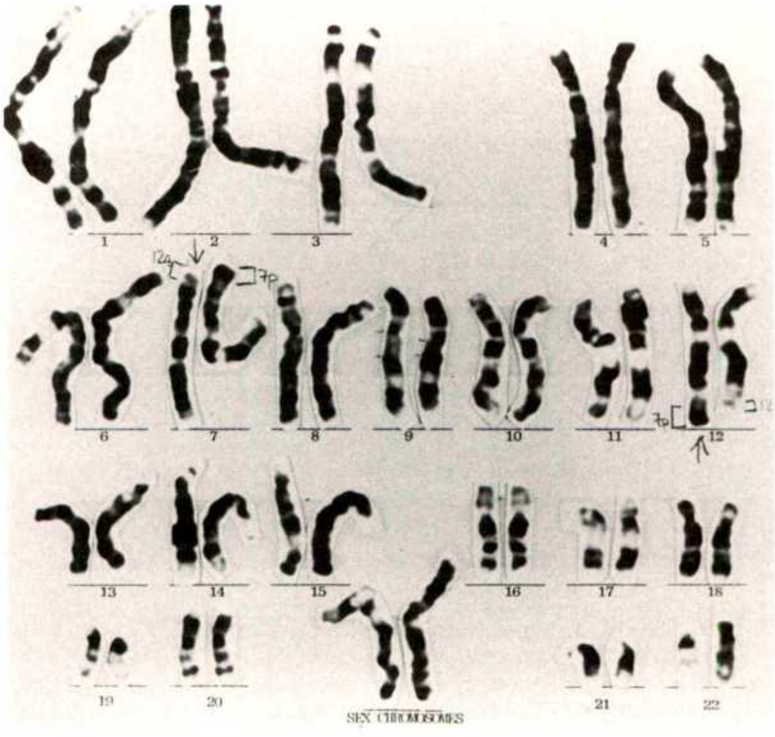

Fig 1. Karyotype analysis of patient.

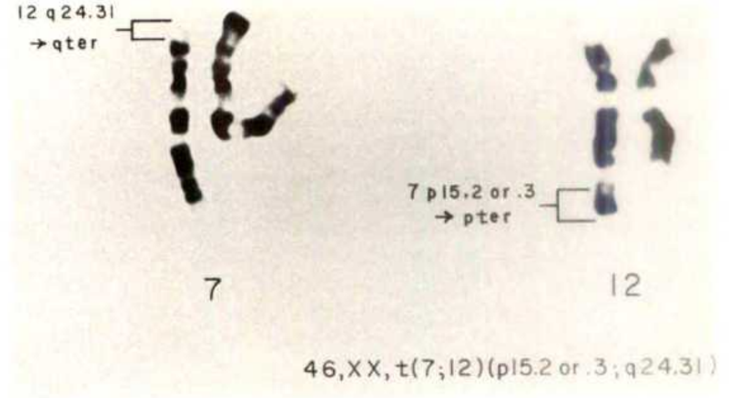

Fig 2. Closer view showing 46, XX, t(7;12) (p 15.2;q24.31) karyotype.

eral blood leukocytes, digested with restriction endonucleases, and size fractionated on agarose gels. The size of genomic DNA fragments hybridizing to human α, β, and γ chain cDNAs was compared with similarly processed samples from normal individuals. When digested with restriction endonucleases EcoR1 or HindIII, DNA from the proband's mother gave hybridizing bands of exactly the same size as DNA from a normal subject. Figure 5 illustrates a representative experiment comparing the proband's mother with a normal individual. Fibrinogen cDNA probes for the α, β, and γ chain genes bind to identically sized fragments in each, suggesting no major alterations of gene structure in the regions detected by the probes. Normally a faintly hybridizing 600 bp EcoR1 fragment from the extreme 3′ end of the alpha fibrinogen gene is present. The absence of this band in both the patient and normal individual suggests the common problem of poor retention of small restriction fragments on nitrocellulose membranes. Identical results were seen with the proband and her daughter.

Following consent of both the patient and the University of Florida Investigational Review Board, she was admitted to the Clinical Research Center for further studies. Thirty units of cryoprecipitate containing 6 g fibrinogen were administered, which increased her plasma fibrinogen level from 80 mg/dL to 340 mg/dL; analysis of subsequent fibrinogen levels determined the plasma fibrinogen half-life to be normal at 96 hours.[24]

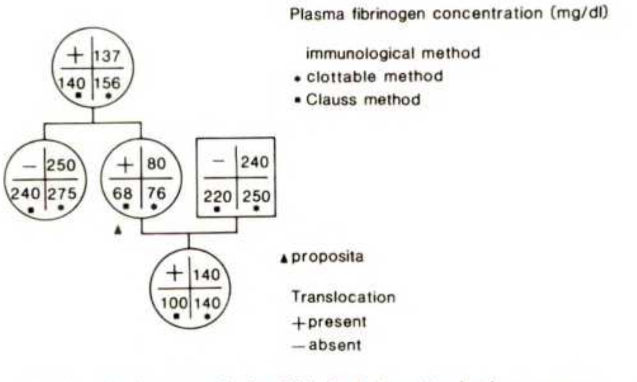

Fig 3. Pedigree analysis of kindred. Females in three generations show both the 7p/12q translocation and hypofibrinogenemia.

She became pregnant. Vaginal spotting began in the fifth week at a time when her plasma fibrinogen level was 71 mg/dL. She was immediately tranfused with cryoprecipitate, 10 U, after which time spotting ceased. She was infused weekly with cryoprecipitate from the fifth to the twelfth week of pregnancy at which time her plasma fibrinogen level was maintained in the range of 105 to 275 mg/dL (averaging 150 mg/dL). From the 12th week to parturition, her plasma fibrinogen level increased to 200 mg/dL without further cryoprecipitate infusion consistent with the increase in fibrinogen concentration experienced in pregnancy. She had no further spotting. She delivered a normal female infant.

One year later, she became pregnant a fourth time. The patient elected not to receive cryoprecipitate infusion. Spotting occurred in the fourth week and she spontaneously aborted in the ninth week at a time her fibrinogen concentration was less than 100 mg/dL. Analysis of the abortus and its blood was unsuccessfully attempted.

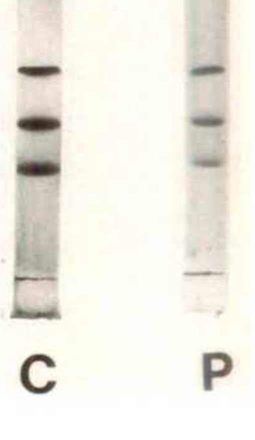

Fig 4. Reduced fibrinogen from the patient shows electrophoretic mobility identical to normal. C = control, P = patient.

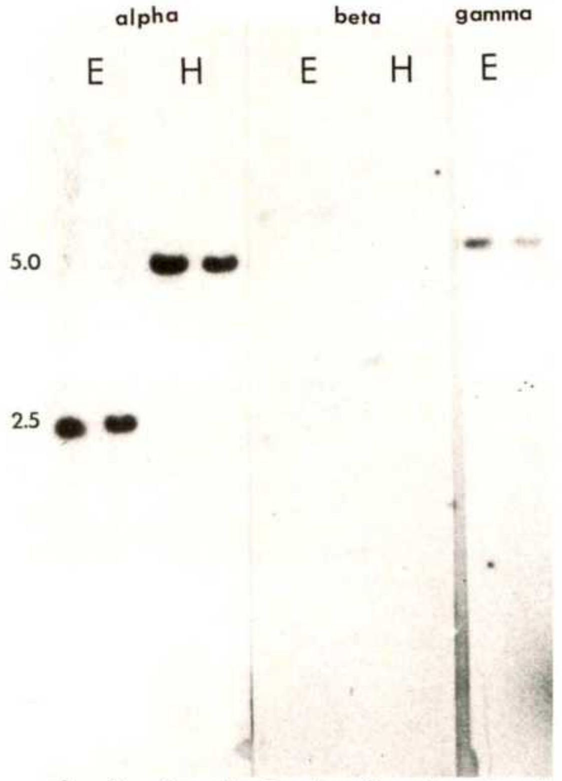

Fig 5. Fibrinogen genes are grossly unaltered in patients with the 7p/12q translocation. Genomic DNA from a normal individual and the proposita with the 7p/12q translocation was digested with restriction endonucleases EcoR1 (columns labeled with large E; lanes A, B, E, F, I, and J) or HindIII (columns labeled with large H; lanes C, D, G, and H) and fractionated on agarose gels. Lanes A, C, E, G, I—proposita; Lanes B, D, F, H, J—normal. Lanes A—D were hybridized to human α fibrinogen cDNA, Lanes E—H with the β cDNA, and Lanes I and J with the γ cDNA. The β and γ hybridizations are lighter than the γ because 50% to 75% fewer counts were added. The size of EcoR1 fragments hybridizing with α, β, and γ probes is 2.5, 6.0, and 5.0 kb, respectively. The size of HindIII fragments hybridizing with α and β probes is 5.0 and 3.3 kb, respectively.

DISCUSSION

The coagulation proteins, particularly fibrinogen and fibrin, seem to play a role in implantation and maintenance of the fertilized ovum in the uterine wall. Evidence to support the hypothesis that fibrinogen and fibrin homeostasis are important in pregnancy are presently supported by clinical observations. At least six publications[9-12,14,25] report hypofibrinogenemia or dysfibrinogenemia associated with spontaneous abortion, including placental abruption. The abortions seen with fibrinogen Metz are restricted to the homozygous case and are not experienced in the heterozygous state. The patient reported by Gralnick and colleagues[11] also experienced successful gestation when functional plasma fibrinogen levels were supported during early gestation by cryoprecipitate infusion. Women congenitally lacking coagulation factor XIII, the enzyme that cross-links fibrin and links fibrin to fibronectin, habitually abort.[13] One pregnancy in a woman lacking factor XIII was successfully carried to term when factor XIII levels were maintained by chronic infusion of cryoprecipitate.[26] Reid and colleagues described abortion in pregnant women who developed acquired afibrinogenemia following envenomation by the Malayan pit viper.[9] There is no evidence to suggest that this kindred also has a dysfibrinogenemia. To exclude exhaustively that possibility, other studies to include immunoelectrophoresis and turbidity studies of fibrin assembly would be necessary. Accordingly, at this time we assume the fibrinogen abnormality is purely quantitative.

Experimental models also emphasize the role of hemostatic proteins in pregnancy. Mouse trophoblasts produce a plasminogen activator. Peak production coincides with the timing of the uterine invasion. If one blocks fibrinolysis with epsilon aminocaproic acid in pregnant rats, fetal wastage increases sixfold. This effect seemed to be dose-related but could be neutralized with plasmin administration.[27]

The observation that fibrinogen genes from our patients appear to be structurally normal is not surprising, since the human α, β, and γ fibrinogen genes have been found linked in a small region of chromosome 4 at bands 4q23-4q32.[28-31] Small structural changes that might affect the levels of expression of one or more genes would not be detected by our studies. Moreover, since cDNA probes used to probe the Southern blots do not span the entire gene, it is possible that structural changes at the 5′ ends of each gene might not be appreciated. The basis for the hypofibrinogenemia in this kindred is thus unclear. The 7p/12q translocation seems to be associated with low or low-normal fibrinogen concentration in each of the observed generations, and it is possible there is a gene on chromosome 7 or 12 whose disruption by the translocation might adversely affect fibrinogen production. Conversely, the translocation could affect other genes important in maintaining pregnancy or have no causal role. Since the proposita successfully sustained a pregnancy when her fibrinogen concentration was supported by cryoprecipitate infusion, it seems less likely that the chromosomal abnormality in and of itself is the cause of her recurrent fetal wastage.

ACKNOWLEDGMENT

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Cytogenetic Studies on Prolymphocytic Leukemia.
II. T Cell Prolymphocytic Leukemia

By Vasantha Brito-Babapulle, Mark Pomfret, Estela Matutes, and Daniel Catovsky

We report chromosome abnormalities in 15 cases of T cell prolymphocytic leukemia (T-PLL). All cases were characterized by clinical, morphological, and membrane marker analysis. The most frequent abnormality was an inv(14)(q11q32) observed in nine cases. The T cell receptor (TCR) α chain gene is localized to 14q11 and the immunoglobulin heavy-chain gene to region 14q32. Four cases also had translocations involving 14q11. Trisomy or multisomy for 8q resulting from an i(8q) or from rearrangements with 8p12 as the breakpoint was observed in nine cases, and a deletion of 6q was found in four cases. Trisomy or partial trisomy for 7q was observed in four cases, of which two had abnormalities of band 7q35 to which the TCR β chain gene is mapped. The expression of Tac antigen, investigated in 27 cases of human T cell leukemia virus I–negative chronic T cell leukemia, which included the 15 cases of T-PLL, showed a good correlation with abnormalities of 7q35. Our studies on chronic T leukemias suggest that inv(14)(q11q32) and trisomy for 8q are abnormalities characteristic of T-PLL.

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PROLYMPHOCYTIC LEUKEMIA (PLL) was first described by Galton et al[1] in a series of patients characterized by marked splenomegaly, high WBC count, and minimal lymph node enlargement. Diagnosis was made by the identification of the prolymphocyte in peripheral blood (PB) films.[1] With the advent of cell marker studies a form of the disease where the leukemic cell is of T cell origin (T-PLL) has been recognized and represents a distinct clinicopathologic entity.[2] Two thirds of T-PLL cases have a CD4+, CD8− membrane phenotype, whereas others may be CD4−, CD8+ or react with CD4 and CD8.[3] The prolymphocytes from 95% of T-PLL cases react strongly with the CD7(3A1) antibody, whereas in adult T cell lymphoma-leukemia (ATLL), Sézary syndrome (SS), and T-chronic lymphocytic leukemia (T-CLL) only 10% to 35% of cases react with this antibody.[4] T prolymphocytes have an irregular nucleus, with peripheral chromatin condensation, a single prominent nucleolus, and a cytoplasm devoid of azurophilic granules.[3] These cells show some morphological differences from B prolymphocytes; a small-cell variant of T-PLL has also been described.[3] T-PLL is an aggressive disease, and the mean survival in a series of 40 patients from our institution was found to be 6 months.[4]

Few cases of T-PLL have been reported with cytogenetic studies.[5-7] We describe chromosome abnormalities in 15 new cases of T-PLL, of which ten had rearrangements involving 14q11 to which the T cell receptor (TCR) α chain gene is mapped,[8] and two of the remaining five had rearrangements of 7q35 to which the TCR β chain gene is mapped.[9]

MATERIALS AND METHODS

Patients. Laboratory studies were carried out on PB mononuclear cells from 15 patients with T-PLL. The diagnosis was based on clinical, morphological, and immunologic features and was confirmed in each one by ultrastructural studies.[3] Serum antibodies to the human T cell leukemia virus (HTLV-I) and reactivity of the leukemic cells with monoclonal antibodies (MoAb) against HTLV-I core proteins p19 and p24 (gift from Dr B.F. Haynes and Dr M. Robert-Guroff), tested after seven days' culture, were negative in all the samples.

Clinical features. These are summarized in Table 1. Briefly, all but two patients (nos. 9 and 12) presented with systemic symptoms (malaise, anorexia, anemia, effusions, skin rash, or lymphadenopathy). Physical examination revealed massive hepatosplenomegaly in 11 patients and generalized lymphadenopathy in nine patients. The total WBC count was markedly raised ($>100 \times 10^9$/L) in 12 patients; in the remaining three patients (WBC, 48 to 50 × 10^9/L), the WBC count tended to increase during the short follow-up. The clinical response to various modalities of chemotherapy (COP, CHOP, M-BACOP, and deoxycoformycin) was poor overall.

Light and electron microscopy. PB examination of May-Grünwald-Giemsa–stained films showed that over 90% of cells had the characteristic features of prolymphocytes,[1,3] namely, a high nucleocytoplasmic ratio, peripheral nuclear chromatin, a prominent vesicular nucleolus, and a cytoplasm devoid of granules (Fig 1, inset). The diagnosis of T-PLL was confirmed in all cases by electron microscopy examination (Fig 1). This technique was particularly useful for the diagnosis of cases nos. 4 and 7, small-cell variant of T-PLL.[3]

Membrane marker analysis. The immunophenotype was assessed by indirect immunofluorescence with a battery of MoAb, and a fluorescein-conjugated F(ab)$_2$ antimouse immunoglobulin was used as second layer. The following MoAb were used: UCHT1(CD3) (gift from Dr P.C.L. Beverley), OKT4(CD4) (Orthoclone, Diagnostics, Westwood, MA), OKT6(CD1), OKT8(CD8) (Orthoclone), OKT17,[10] 3A1(CD7),[11] and anti-Tac (CD25) against the receptor for interleukin-2 (IL-2)[12] (gift from Dr T. Uchiyama). The reactivity with anti-Tac was assessed on fresh leukemic cells and also after 72 hours' culture at 37°C in RPMI 1640 medium (GIBCO, Grand Island, NY) without added mitogens. E rosettes were performed by using standard techniques, and the presence of terminal deoxynucleotidyl transferase (TdT) was tested on cytocentrifuge slides fixed in acetone by indirect immunofluorescence using a rabbit anti-TdT antibody.[13]

Cytogenetics. Mononuclear cells were seeded at a concentration of 1.5 to 2.0 × 10^6 cells/mL in medium RPMI 1640 supplemented with 20% fetal calf serum. The cells were cultured in the presence of conditioned medium containing IL-2 (1 mL/10 mL culture) with

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Table 1. Clinical Data on 15 T-PLL Cases

Case	Age/Sex	Spleen	Lymph Nodes	WBC (× 10⁹/L)	Response to Treatment	Survival	Follow-up
1	90/M	++	–	450	NR	6 wk	
2	60/M	++	–	500	NR	3 mo	
3	83/F	++	–	114	NR	2 mo	
4•	77/M	–	–	104	PR	3 wk	
5	48/M	++	++	328	NR	Alive	3 yr
6	69/M	–	++	400	NR	4 mo	
7•	46/M	–	++	48 (rising)	Alive—not treated		18 mo
8	58/M	++	++	400	PR	6 mo	
9	77/F	–	–	48 (rising)	NR	Alive	5 mo
10	73/F	++	++	800	PR	Alive	5 mo
11	56/M	++	++	495	No follow-up data		
12	80/F	+	–	50 (rising)	Alive—not treated		3 mo
13	33/M	++	++	207	No follow-up data		
14	58/F	++	+	346	No follow-up data		
15	62/M	+	+	96.7	Recently diagnosed		

In all cases except no. 5, chromosome studies were performed at diagnosis before treatment.

Abbreviations: NR, no response; PR, partial response.

•Small-cell variant.[3]

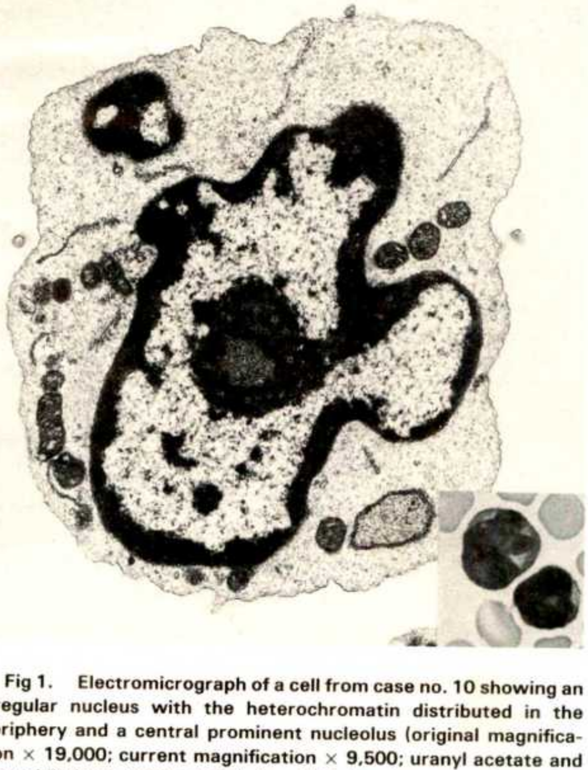

Fig 1. Electromicrograph of a cell from case no. 10 showing an irregular nucleus with the heterochromatin distributed in the periphery and a central prominent nucleolus (original magnification × 19,000; current magnification × 9,500; uranyl acetate and Reynold's lead nitrate stain). (Inset) Light microscopy from a PB film from a T-PLL showing cells with a high nucleocytoplasmic ratio, irregular nucleus with a single vesicular nucleolus, and a cytoplasm devoid of azurophil granules (original magnification × 1,400; current magnification × 700).

phytohemagglutinin (PHA) at 0.2 μg/mL only and PHA (0.1 μg/mL) together with 12-0-tetradecanoylphorbol-13-acetate (phorbol ester) at 10^{-7}/mL as mitogens for 3, 5, and 7 days. Cells were harvested after incubation with 0.05 μg/mL colcemid (GIBCO) for one hour, followed by hypotonic treatment, and fixed in one part glacial acetic acid to three parts methanol. Trypsin Giemsa banding was carried out on air-dried slides by standard methods.

RESULTS

Cell markers. The immunophenotyping analysis revealed that cells from all the cases had a mature postthymic T cell phenotype (TdT–, CD1–, E rosettes+, CD7+, OKT17+); 11 of them were CD4+, CD8–; three coexpressed CD4 and CD8; and one was CD4–, CD8+. The CD3 antigen was not expressed in cells from two patients (case nos. 3 and 10), and CD25 (Tac) was expressed in prolymphocytes of four cases (case nos. 2, 3, 6, and 13) (data summarized in Table 2).

Cytogenetics. Clonal chromosome abnormalities were observed in 14 out of 15 cases of T-PLL, and these are summarized in Table 3. Region 14q11 was involved in various rearrangements in case nos. 1, 4, 6, 8, 10, 11, 12, 13, 14, and 15. An inv(14)(q11q32) was observed in nine cases, although in one of them (case no. 6) it was in a minor subclone (scl_2) (see Table 2, Fig 2B, C, D, E, F). The major subclone (scl_1) of that case had a t(13;14)(q34;q11) instead of inv(14). A t(X;14)(p11;q11) was present in addition to an inv(14) in case no. 10 (Fig 2E). In case no. 11 a t(14;17)(q11;q24) was present in a subclone (scl_1) in addition to the inv(14) (Fig 2F). Case no. 12 had rearrangement between chromosome 11 and both homologues of 14 that resulted in a t(11;14)(14;14)(p13;q11q32;q11) (Fig 2G). Trisomy or partial trisomy for 7q was observed in four cases (nos. 2, 3, 4, and 5), and in two of these (nos. 2 and 3) the

Table 2. Immunophenotype of 15 Cases of T-PLL

Case	CD3	CD4	CD8	Tac
1	+	+	−	−
2	+	−	+	+
3	−	+	−	+
4	+	+	−	−
5	+	+	+	−
6	+	+	−	+
7	+	+	+	−
8	+	+	−	−
9	−	+	−	−
10	+	+	−	−
11	+	+	−	−
12	+	+	−	−
13	+	+	+	+
14	+	+	−	−
15	+	+	−	−

All cases were TdT−, CD1−, CD5+, CD7+, E rosettes +/−.

Table 3. Chromosome Abnormalities in 15 Cases of T-PLL

Case No.	No. of Abnormal Cells	Karyotype
1	5/8	46,XY,−8, idic(8q),12q+,inv(14)(q11q32)
2	5/5	46,Xq+Y,−8,inv dup(7)(q21 → 35),t(7;15)(q35; q13),+i(8q),dup(X)(q12 → qter) *(Fig 2A)*
3	3/6	42,X,−Y,1p+,del(6)(q21),t(?;7)(7;?)(?;p22;q35;?)
4	9/15	46,XY,−7,−8,+i(7q),+t(8;20)(q13;p13),inv(14)(q11q32), t(14;21)(p11;q11) *(Fig. 2B)*
5	18/18	44,XY,−7,−8,1p+,1q−,4p+,int del(6)(q15q22) inv dup(7)(q22 → q33),i(8q)
6	42/50	46,XY,−8,del(6)(q21),+t(8;8)(p12; q11)t(11;12)(q21;q24),der13t (13;14)(q34;q11) *(Fig 2C)*
	5/50	45,X,−Y,−8+t(8;8)(p12;q11), t(11;12)(q21;q24), inv(14)(q11 q32) *(Fig 2C)*
7	8/20	47,XY,+22,t(3;11)(q25;q13)
8	30/30	49,XY,−8,+i(8q),+i(8q),+i(8q), 10q−,inv(14)(q11q32),+minute *(Fig 2D)*
9	25 cells analyzed	Random abnormalities
10	18/20	46X,−X,−9,+t(X;9)(p24;p11), +der(X)t(X;14)(p11;q11),t(5;6)(q13;q27),inv(14)(q11q32) *(Fig 2E)*
11	7/26	44,XY,−8,−13,−17,+t(8;8)(p12; q11)der(17)t(14;17)(q11;q25),inv (14)(q11q32) *(Fig 2F)*
	19/26	44,XY,−8,−13,−17,−21,int del(6)(q21q24)+t(8;21)(p12; q11),+t(8;21)(p12;q11),inv(14)(q11q32) *(Fig 2F)*
12	62/66	45XX,−14,der11t(11;14)(14;14)(p13;q11 q32;q11),20q+ *(Fig 2G)*
13	10/10	45,XY,−22,i(8q),inv(14)(q11q32) psu dic(16)t(16;16)
14	6/10	43,X,−X,−7,−16,−17,−22,+3, +M,inv(3)(q22q29),i(8q+),inv (14)(q11q32)
15	20/20	45,XY,−22,i(8q),inv(14)(q11q32)

chromosome rearrangements affected 7q35 (Fig 2A). Trisomy or partial trisomy for 8q was observed in eight cases (nos. 1, 2, 5, 6, and 11), and in one case (no. 8) there was multisomy for 8q due to the presence of three copies of i(8q) (Fig 2D). Chromosome 6 was involved in abnormalities in five cases. Four cases (nos. 3, 5, 6, and 11) had deletions, and one (case no. 10) had a translocation t(5;6)(q13;q27). Case 9 had random abnormalities in a few of the 25 cells analyzed.

DISCUSSION

Ten out of the 15 cases of T-PLL studied had chromosome abnormalities involving 14q11, which is the chromosomal band to which TCR α chain gene is localized.[8] Nine had an inv(14)(q11q32), although in one (case no. 6) the abnormalities were secondary with a t(13;14)(q34;q11) in the scl_1 and inv(14)(q11q32) in the scl_2. In case no. 12 translocations between chromosome no. 11 and both homologues of no. 14 resulted in the juxtaposition of 11p13 with 14q11 and 14q11 with 14q32. Thus, in ten cases 14q11 and q32 were made adjacent to each other. The immunoglobulin heavy-chain gene and the AKT-1 oncogene are localized to 14q32.[14,15]

Although abnormalities of chromosome no. 14 with q11 as the breakpoint have been described in many T cell disorders, these are usually translocations between no. 14 and other chromosomes. For example, in T cell acute lymphocytic leukemia (T-ALL) a t(11;14)(p13;q11) has been described in six cases,[16-18] t(10;14)(q24;q11) in four cases,[19,20] t(8;14)(q24;q11) in four cases,[16,21] and t(9;14)(q34;q11) in one case.[15] We are not aware of tandem translocations or inversions involving 14q11 and 14q32 in T-ALL. A t(9;14)(q34;q11) and t(10;14)(q24;q11) have also been described in a case of SS[22] and in a T lymphoma, respectively,[19] and a t(14;17) has been described in a chronic T cell leukemia.[23]

Inversions resulting in the adjacency of 14q11 with 14q32 are not very frequent in many of the T cell disorders. Because the majority of chromosome abnormalities are not absolutely specific, the significance of such an abnormality can be determined only by its incidence in a given disease. Inv(14)(q11q32) was found in 60% of cases in the present series of T-PLL and has been described in two of 21 cases of T lymphoma,[22,23] in another case of T-PLL[6] in two of three cases described as chronic T leukemia,[24] and seven of eight cases described as T-CLL.[25] The morphology of the leukemic lymphocytes was not described in the latter two studies. The inv(14)(q11q32) in the cell line SUP-T1 derived from a T lymphoma results from a site-specific recombination event between an immunoglobulin heavy-chain variable gene (IgV_H) and a TCR α chain joining segment (TCRαJ), which gives rise to an Ig-TCR hybrid gene. This suggests that there are common signal sequences present in both TCRα and IgH

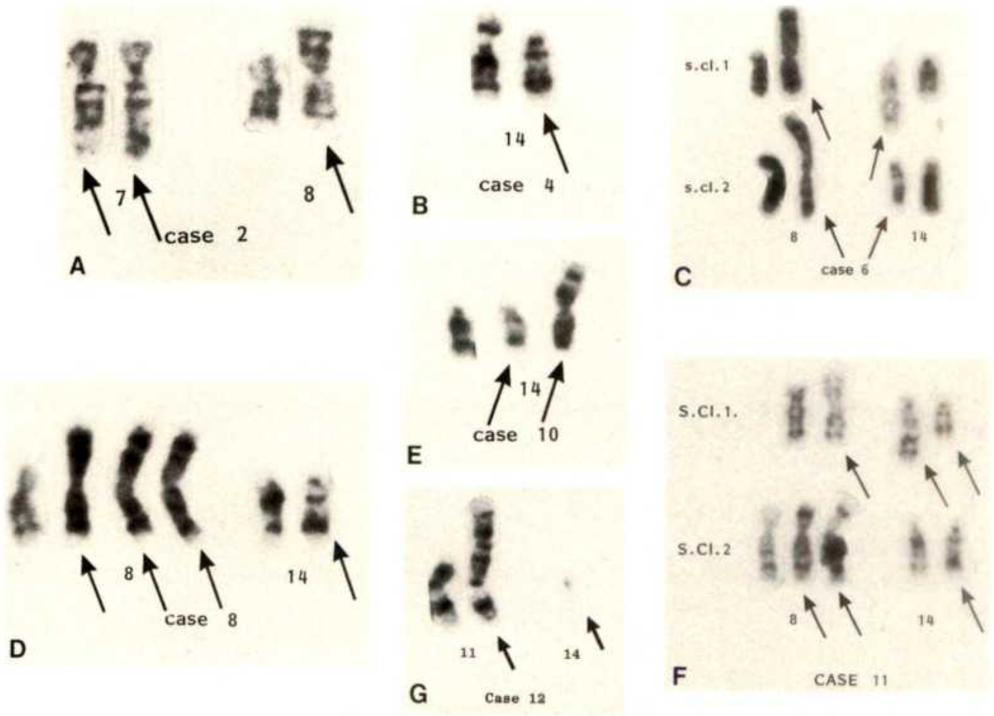

Fig 2. Abnormalities involving 14q11 or 7q35 and/or abnormalities of chromosome 8 resulting in trisomy or multisomy for 8q in case nos. 2 (A), 4 (B), 6 (C), 8 (D), 10 (E), 11 (F), and 12 (G).

loci that make them susceptible to the same recombinases.[26] However, the possible involvement of an oncogenic DNA sequence from 14q32 in the inversions in T-PLL cannot be excluded and has yet to be investigated.

Trisomy, partial trisomy, or multisomy for 8q was observed in nine of 15 patients in the present series. Trisomy for 8q has been described in six of 12 chronic T lymphoid malignancies by Zech et al.[25] Of these, one out of four with SS had trisomy for the whole chromosome 8. Of the remaining eight cases described as T-CLL, five had trisomy or partial trisomy for the q arm of no. 8 by formation of an i(8q) or a t(8p−;8q). All cases of trisomy or partial trisomy of 8q in the present series were also due to an i(8q) or t(8p−;8q), except case no. 11 where two copies of t(8;21)(p12;q11) were present in addition to a normal no. 8 in a subclone. Whether the increased dosage of *c-myc* localized on 8q24[27] has any significance in T-PLL remains to be seen. The high incidence of inv(14)(q11q32) (seven of eight cases) and i(8q) or t(8p−;8q) (five of eight cases) in the series reported under the diagnosis of T-CLL by Zech et al[25] is similar to that of our series of T-PLL. In the absence of morphological information it is not possible to exclude a diagnosis of T-PLL in some of the cases described by Zech et al.[25] We use the term T-CLL for a proliferation of T cells (often T γ cells) with the morphology of large granular lymphocytes and a more benign clinical evolution than T-PLL.[28] Six cases of T-CLL investigated in our laboratory did not have inv(14)(q11q32) or trisomy for 8q.

Trisomy or partial trisomy for 7q was observed in four cases, and it is of interest that this is a common abnormality in ATLL, a disease associated with HTLV-I with an aggressive clinical course.[29] Of the four cases with chromosome no. 7 abnormalities, two had rearrangements affecting 7q35 to which the TCR β chain gene is localized.[8]

In a previous study we observed that in HTLV-I–negative T cell disorders (T-PLL, SS, T-CLL) those with chromosome abnormalities involving 7q35 showed a correlation with Tac (T cell growth factor [IL-2] receptor) antigen expression in the absence of mitogenic stimulation.[30] At present, 27 of these T cell malignancies have been tested for Tac expression, six of which were positive (Table 4). Four had rearrangements of 7q35 and two of 14q11. The correlation between abnormalities of 7q35 and Tac expression was found to be highly significant ($P = .015$, Fisher exact test).

Another chromosome abnormality quite common in T cell disorders is a deletion or interstitial deletion of chromosome no. 6.[30,31] This was also observed here in three cases of T-PLL. The protooncogene *c-myb* has been mapped to 6q22-24,[32] and recent evidence indicates that *c-myb* may play a role in thymic lymphocyte differentiation.[33]

Our studies on T-PLL have revealed that, although chromosome abnormalities common to other T cell disorders such as 6q- trisomy 7q, and translocations involving 14q11 are present, inv(14)(q11q32) and trisomy for 8q are particularly characteristic of this disease.

A detailed clinical, immunologic, and ultrastructural analysis of the T cell leukemias will be conducive to a more objective classification of these disorders,[34] with the possibility of confirming the striking association between inv(14)(q11q32) and T-PLL reported here.

Table 4. Correlation of Tac Expression With Chromosome Rearrangements of 14q11 and 7q35 in 27 Cases of Chronic T Leukemia (HTLV-I-)

Disease	No. With Abnormalities of 14q11 and 7q35	Type of Rearrangement	14q11-12	7q35	Tac
SS	2/6	Translocation	–	+	+
		Translocation	–	+	+
T-PLL	12/15	Inversion (1)	+	–	–
		Translocation/inversion duplication(2)	–	+ +	+
		Translocation (3)	–	+	+
		Inversion (4)	+	–	–
		Translocation/inversion (6)	+/+	–	+
		Inversion (8)	+	–	–
		Inversion/translocation (10)	+ +	–	–
		Inversion/translocation (11)	+/+ +	–	–
		Translocation (12)	+ +	–	–
		Inversion (13)	+	–	+
		Inversion (14)	+	–	–
		Inversion (5)	+	–	–
T-CLL	0/6		–	–	–

All four cases with an abnormality of 7q35 were Tac+, and only two of ten cases with an abnormality of 14q11 were Tac+. Of the 21 Tac- cases, eight had abnormalities of 14q11, and none had abnormalities of 7q35.

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Delineation of a Novel Pre-B Cell Component in Plasma Cell Myeloma: Immunochemical, Immunophenotypic, Genotypic, Cytologic, Cell Culture, and Kinetic Features

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A novel pre-B cell component in direct and cultured myeloma bone marrow material has been delineated by using immunochemistry and flow cytometry techniques. Our phenotypic studies suggest a novel hybrid expression of pre-B and plasma cell antigens with coexpression of cytoplasmic μ, common acute lymphoblastic leukemia antigen, terminal deoxynucleotidyl transferase, and plasma cell antigens (PCA-1 and PC-1). This suggests that myeloma pre-B-like cells are aberrant malignant cells and not normal pre-B lymphocytic counterparts. With the advantage of a pure and stable source of these cells from M3 culture to allow molecular characterization, we performed one- and two-dimensional gel electrophoresis and Western blotting. We found that the cytoplasmic μ in myeloma pre-B-like cells has a molecular weight of 74,000 daltons and an isoelectric point of 6.3 and that it is strikingly homogeneous and discrete in size and charge compared with standard secretory μ, which suggests an aberrant, mutant, or monoclonal form of μ. Monoclonality was further evidenced by heavy- and light-chain immunoglobulin gene rearrangements demonstrated with J_H and C_κ probes. We also established that this novel myeloma pre-B component is a major proliferative element as determined by double-labeling experiments with phenotype coupled to labeling/proliferative indexes. Our stimulatory studies indicate some capacity of these cells to mature on exposure to phorbol esters. These myeloma pre-B cells may represent the stem cell or self-renewal component in myeloma. Our establishment of these cells in long-term culture offers a considerable asset in studying the immature cells, which may be critical to the immortalization of myeloma.

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MULTIPLE MYELOMA is understood to be a malignancy of the plasma cell, the most mature cell in the B cell series. As a tumor of the "most differentiated" immunoglobulin-producing cells, it is the paradigm of monotypic immunoglobulin expression and associated with the most mature antigen expression: cytoplasmic immunoglobulin-positive (CIg+), surface immunoglobulin-negative (SIg−), plasma cell-associated and plasma cell antigen-positive (PCA-1+, PC-1+), pan-B-negative (B1−, B2−, B4−), Ia−, common acute lymphoblastic antigen-negative (CALLA−), (Tdt−).[1-4] Interestingly, circulating monoclonal B lymphocytes occur in myeloma, indicating that B lymphocytes are an integral part of the myeloma clone.[5-8] Recently, it has been found that in some myeloma patients, antigens normally found at a very early stage of B cell differentiation (eg, CALLA) are expressed by these monoclonal peripheral blood lymphocytes as well as by lymphoid cells admixed with marrow plasma cells.[5-8] More recently, some of these immature B cell phenotypes (eg, CALLA+) have also been noted in bone marrow plasma cells of myeloma patients.[9-12] The occurrence of CALLA expression in both myeloma cells and B lymphocytes has been identified as a clinically significant finding portending a poor prognosis.[10-12]

In the present study, we sought to extend our observations of cells with an immature B phenotype in myeloma bone marrow by further analysis of their immunochemical, immunophenotypic, genotypic, cytological, and kinetic properties. By these means we identified one myeloma patient with a significant (>20%) pre-B cell-like (CIgM+, CALLA+, SIg−) component at initial presentation and in subsequent cell culture. Two additional myeloma cell cultures also proved to have substantial pre-B cell components suitable for study. A key development in this study was the emergence of the sustained pre-B cell growth in culture using the recently devised M-3 culture medium.[13] An ongoing difficulty in the study of the immature phenotypes in myeloma is their frequent occurrence as a minority component and consequent difficulty in separation from other components. The pre-B cell lines proved pivotal in supplying an ongoing, nearly pure source of pre-B cells for both immunochemical and molecular characterization.

By means of immunocytochemistry and flow cytometry, we delineate the occurrence of a cell population with a pre-B-like phenotype with coexpression of multiple B antigens: CIgM+, SIg−, BI+, B4+, CALLA+, Tdt+, PCA-1+, PC-1+. This phenotype suggests a novel association between pre-B antigens and plasmacytic antigens and indicates that these are pre-B myeloma cells, not normal pre-B lymphocytic counterparts. By means of one- and two-dimensional (1-D, 2-D) gel electrophoresis and Western blotting of pre-B-like myeloma cells, we have characterized the structural and chemical properties of the stage-specific cytoplasmic μ. We note the striking homogeneity in size and charge of malignant compared with normal μ, which suggests that malignant μ is an aberrant or mutant μ. Monoclonality was further evidenced by heavy-and light-chain immunoglobulin gene rearrangements. Our molecular weight (mol wt) and isoelectric point (pI) determinations represent the first molecular characterization of a human pre-B cell μ. To date

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2-D gel fingerprinting of pre-B cells has been performed in animal models of myeloma but not in humans.[14] By the use of double-labeling experiments using phenotype coupled with a labeling/proliferative index, we determined that the novel pre-B–like component is a major proliferative element in these myelomatous tumors.

MATERIALS AND METHODS

Patient and Tissue Selection

From immunohistochemical assessment of 21 direct myeloma marrow aspirates and eight human myeloma cell lines, we previously identified four myeloma patients and six cell lines with CALLA+ myeloma cells.[10] Among these four CALLA+ myeloma patients, one patient had a significant (>20%) pre-B cell (CIgM+, CALLA+, SIg−) component at initial presentation that became dominant in subsequent cell culture (patient A, culture A, Table 1). The initial marrow in patient A also had many plasma cells with immature B antigens (eg, CALLA+). Two additional myeloma cell cultures (cultures B and C) also proved to have prolific pre-B cell components suitable for study. With B and C, the initial direct marrow material had <1% pre-B cells, and the monoclonal plasma cells were mature CALLA−. In both these cases the pre-B–like cells emerged as a dominant feature only in subsequent long-term culture. Both the direct bone marrow and cell culture material offered the opportunity to study myeloma-derived pre-B cells in considerable immunochemical and cytological detail.

These patients (A, B, and C, Table 1) all had active myeloma at the time material was obtained for study. Patient A was a 48-year-old man who had IgGκ CALLA-positive myeloma stage IIIA at diagnosis, with extensive lytic bone disease and hypercalcemia. He responded poorly to multiple therapies and died of CNS disease after 6 months. Patient B was a 46-year-old man with IgGλ myeloma evaluated 1 year after diagnosis at the time of relapse (IgG, 6.3 g/dL; serum β_2-microglobulin, 10.2 mg/L) on combined alkylator and anthracycline therapy. He failed to respond to the VAD (vincristine, adriamycin, and dexamethasone) regimen and died 6 months later of progressive disease. Patient C was a 52-year-old man with IgAλ myeloma, stage IIIA at diagnosis, who was studied serially while receiving treatment. He responded to induction therapy with combined alkylators and anthracycline. Having been in remission and not receiving therapy for 3 years, he has recently relapsed.

Cell Culture Methods

In vitro colony culture of myeloma cells was carried out as previously reported.[15] In addition, a more extended liquid culture was carried out with a new M-3 medium.[13] The latter medium incorporates the L-15 medium as its base with additional detoxification agents (eg, polyvinylpyrrolidone-360, methyl cellulose catalase, reduced glutathione, selenous acid, and ascorbic acid) and growth factors (eg, insulin, transferrin, hydrocortisone, and additional glutamine).[13,16] After 3 months, cell cultures A, B, and C were well established and could be maintained using M-3 medium supplemented with fetal calf serum (FCS). Aliquots from each passage were frozen for study.

Immunologic Marker Studies

Immunoperoxidase Cytospin Typing

Cytocentrifuge (Shandon Southern Instruments, Sewickley, PA) slide preparations of the myeloma cell material were used for immunologic evaluation. Biotin-avidin conjugates and horseradish peroxidase labeled with diaminobenzidine tetrahydrochloride (DAB) as the detection agent were used, as previously reported.[17] Briefly, cytocentrifuge slides were fixed in acetone at 4°C for ten minutes and air-dried at room temperature. Mouse monoclonal antibody was applied for 30 minutes to detect human cell surface (and/or cytoplasmic) antigen. Mouse ascitic fluid (Bethesda Research Laboratories, Gaithersburg, MD) was applied to one slide in each run instead of the primary antibody as a negative control.

After a phosphate-buffered saline (PBS) wash, a second-stage reagent, biotin-conjugated $F(ab')_2$ goat antimouse IgG (Tago Diagnostics, Burlingame, CA) was added and followed by a 20-minute incubation and a PBS wash. The cytocentrifuge slides were next incubated with avidin-D conjugated with horseradish peroxidase (Vector Laboratories, Burlingame, CA). After another 20-minute period, the slides were again washed with PBS. Next, each slide was incubated with a DAB solution consisting of 3 mg DAB/mL PBS along with 0.01 mL 30% H_2O_2/mL PBS. After the five-minute incubation in DAB solution, the slides were washed with PBS for three changes and rinsed in two changes of distilled water. The slides were incubated in a copper sulfate solution for five minutes, rinsed with distilled water, and counterstained with methylene blue for five minutes. After air drying the slides were coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ).

The first stage included monoclonal antibodies to the following B cell antigens (B1, B2, B4; Coulter Immunology, Hialeah, FL), (Leu 12, Leu 14; Becton Dickinson, Mountain View, CA), immunoglobulins (κ, λ, μ, γ, α, δ; Becton Dickinson), plasma cell antigens (PCA-1, Coulter), (PC-1, kindly supplied by Drs Kenneth Anderson and Lee Nadler, Dana-Farber Cancer Institute, Boston),[2] CALLA, and HLA-DR (Ia), T cell antigens (Leu 1-9), transferrin receptor (TRF), and myelomonocytic antigens (LM1, LM3, LM5) (all obtained from Becton Dickinson). Anti–interleukin-2 (anti–IL-2) receptor monoclonal was obtained from Coulter.

Nuclear Tdt activity was determined by using a rabbit anticalf Tdt (Bethesda Research Laboratories [BRL], Rockville, MD) followed by biotin-conjugated $F(ab')_2$ goat antirabbit IgG (Vector) as previously described.[18]

Flow Cytometric Analysis

Flow cytometry. Flow cytometry was conducted with fluorescent antibodies analyzed by a Coulter EPICS V flow cytometer (EPICS Division, Coulter Immunology, Hialeah, FL).[19] Data obtained by flow cytometry was analyzed by using the MDADS (Multiparameter Data Acquisition and Display System) "Immuno" program; the coplot display of composite histograms was created by using the EASY 88 (Extended Analysis System with Intel 80/88 Microprocessor) analysis system (see Figs 2 and 3).

Surface membrane indirect immunofluorescence. With a previously described method[17,19,20] surface antigens were detected by using indirect immunofluorescence (IF). Briefly, myeloma cells were washed in PBS (pH 7.4). Before staining, the cells were incubated at 37°C for 30 to 60 minutes to elute immunoglobulin attached by Fc receptor binding.[21] After staining, the unbound conjugate was removed by gradient centrifugation over fetal calf serum (FCS). Trypan blue dye exclusion was used to establish viability. The myeloma cells were then incubated with the same battery of monoclonal antibodies as before for 30 minutes at 4°C (except for Leu 6, Leu 7, LM3, LM5, and Tdt). Then a second-stage goat antimouse IgG–fluorescein isothiocyanate (FITC) conjugate was applied and the cells submitted for flow analysis.

Cytoplasmic IgM and other antigens by direct IF. Using a 0.1 mol/L KCl "swelling buffer" as suggested by Loftin et al[22] with minor modification, we detected cytoplasmic μ, CALLA, and B cell antigens by direct immunofluorescence. Briefly, after a five-minute incubation with swelling buffer, we added 0.5 mL cold absolute methanol to fix the cells and followed this by incubation in an ice

Table 1. Immunophenotypes

		B Cell Antigens													T Cell		Others			
	Cell Type	κ	λ	μ	γ	α	δ	B1	B2	B4	Leu 12	Leu 14	PCA	PC	Leu 1-9	M/G	CALLA	Tdt	Ia	TRF
Patient A	Plasma cells	+	0	0	+	0	0	+	0	+	+	±	+	+	0	0	+	Some	+	+
Marrow	Lymphoid/pre-B cells	0	0	+	0	0	0	+	0	+	+	±	±	±	0	0	+	+	+	+
Initial	Plasma cells	+	0	0	+	0	0	+	0	+	+	±	+	+	0	0	+	Some	+	+
Culture A	Lymphoid/pre-B cells	0	0	+	0	0	0	+	0	+	+	±	±	±	0	0	+	+	+	+
Long-term culture A	Lymphoid pre-B cells	0	0	+	0	0	0	+	0	+	+	±	±	±	0	0	+	+	+	+
Culture B	Lymphoid pre-B cells	0	0	+	0	0	0	+	0	+	+	±	+	+	0	0	+	±	+	+
Culture C	Lymphoid pre-B cells																			
Initially		0	0	+	0	0	0	+	±	+	+	±	±	+	0	0	+	+	+	+
Later		0	0	+	0	0	0	±	0	+	+	±	0	+	0	0	+	+	+	+
TPA		0	0	+	0	0	0	±	0	±	±	±	±	+	0	0	0	ND	+	+
Normal plasma cells		+	0	0	+	0	0	0	0	0	0	0	+	+	0	0	0	0	0	±
Normal, pre-B lymphoid cells		0	0	+	0	0	0	+	0	+	+	+	0	0	0	0	+	+	+	+

Abbreviations: M/G, monocyte/granulocytic antigens as detected by anti–Leu M1, -Leu M3, -Leu M5, and -MY7; ND, not done; +, positive; ±, faintly positive; 0, negative.

bath for 30 minutes. After centrifugation and resuspension the fixed cells were incubated with FITC-conjugated goat antihuman IgM at 4°C for 30 minutes, washed, and resuspended. Fixed cells reacted with FITC-conjugated goat antimouse IgG served as controls for nonspecific binding. B antigens were also evaluated by indirect IF after the addition of swelling buffer; this was followed by methanol fixation.[22]

Gel Electrophoresis and Immunoblotting to Characterize Cytoplasmic μ

Gel Electrophoresis

By using published methods[23,24] cells for isoelectric focusing (IEF) analysis were lysed with IEF lysis buffer (including 9.5 mol/L urea, 2% NP-40, 5% β-mercaptoethanol, 1% LKB ampholines 3.5 to 10 in distilled water). After 30 minutes' incubation, douncing, and centrifugation, the lysate was applied to IEF rod gels.[23,24] We generally used 150 μL of lysis buffer for each 12 million cells. Rod gels were made, prefocused, and run according to O'Farrell[23,24] for a total of 10,000 volt hours (Vh). Sample rods from IEF were then incubated for 30 minutes in a sodium dodecyl sulfate (SDS)–sample buffer (including 0.06 mol/L Tris-HC1 [pH 6.8], 2% SDS, 5% β-mercaptoethanol, and 10% glycerol in distilled H_2O). The IEF rods were then subjected to SDS polyacrylamide gel electrophoresis (PAGE) using 1.5-mm-thick slab gels. The separating gel was 12% polyacrylamide with a 5% stacking gel.[25] The rods were oriented with the basic end to the left and anchored with 1% agarose in SDS–sample buffer with bromphenol blue. Electrophoresis was carried out overnight (16 to 18 hours) at 8 to 10 mA/gel in a Bio-Rad Protean II multicell chamber. After completion (indicated by the bromphenol blue), the gels were fixed in 45%:10% methanol:acetic acid solution and stained with Coomassie brilliant blue to nonspecifically visualize proteins. Alternatively, some gels were electroblotted to nitrocellulose paper for further immunologic characterization.

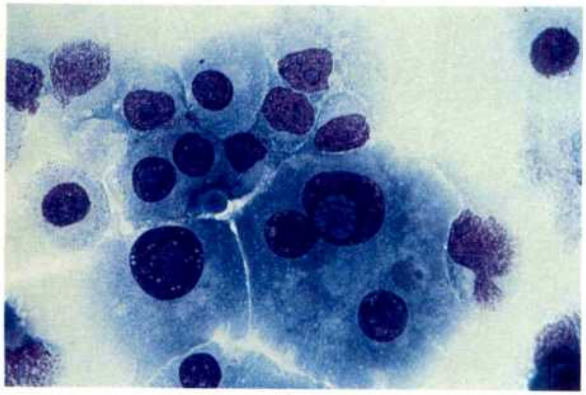
Electroblotting (Western Blotting) and Immunoblotting

Proteins were transferred from the gel onto nitrocellulose paper as previously described[26] at 4°C and 0.35 A for two hours in transfer buffer in a Bio-Rad Trans-blot Electrophoresis Transfer cell. After transfer nitrocellulose sheets were equilibrated in PBS–0.06% Tween 20 for two to three hours. Immunoblotting followed the suggested procedures of Tracy et al[27] and the bovine lactotransfer technique optimizer (Blotto) method of Johnson et al.[28] Nonspecific binding sites were blocked overnight with 5% nonfat milk (NFM) in PBS-Tween.[28] Primary antibodies included rabbit antihuman IgM/κ and λ (Dako, Copenhagen) in PBS with 2% bovine serum albumin (BSA). Washing used successive incubations in 0.1% and 0.5% Triton X-100–PBS–Tween, 0.5 mol/L NaCl–PBS–Tween, and PBS-Tween, all with 5% NFM. The secondary antibody was a peroxidase-conjugated goat antirabbit IgG ($F[ab']_2$) (Cappel Laboratories, Cochranville, PA). This was followed by washes as described earlier and two washes with PBS-Tween without NFM. Peroxidase binding was detected by using 4-chloro-1-naphthol dissolved in cold methanol plus cold 30% H_2O_2 in PBS-Tween.

Reagents

Purified IgM standards were obtained from Calbiochem-Behring (San Diego) and Jackson Immunoresearch Laboratories, Inc, (Avondale, PA). Ampholines were obtained from LKB (Gaithersburg, MD). The other reagents for electrophoresis and immunoblotting were all obtained from Bio-Rad (Richmond, CA). All the buffers and solutions for 2-D gel electrophoresis were made as stated

Fig 1. **Morphological and immunologic features of admixed plasma cells and lymphoid cells, direct marrow aspirate, and cytospin culture preparation. (A) Note large atypical (multinucleated and nucleolated) and typical plasma cells admixed with small nucleolated lymphoid cells. Patient A, Wright's stain, marrow aspirate, original magnification × 1,000; current magnification ×420. (B) Same marrow aspirate as A. Note the large plasma cell with cytoplasmic vacuolation (Mott cell formation) and nuclear inclusion (Dutcher body). The small nucleolated lymphoid cells appear nestled within lacunae of the large plasma cell. Patient A, Wright-stained marrow aspirate, original magnification ×1,000; current magnification ×420. (C) Note the presence of cytoplasmic μ (brown coloration) in small lymphoid cells and the absence of μ in large plasma cells. One μ+ lymphoid cell occurs within a lacuna. Patient A, anti-μ antibody, BA-HRP method, cytospin preparation of marrow aspirate. (D) Note the presence of κ in plasma cells and the absence in immature lymphoid cells. λ Expression was absent in both populations (not shown). Patient A, anti-κ antibody, BA-HRP, cytospin marrow. (E) Note the CALLA expression in both large and small, lymphoid and plasma cell components. Patient A, anti-CALLA antibody, BA-HRP method, cytospin marrow. (F) Note the strong nuclear Tdt expression in smaller lymphoid cells and sparse expression in plasma cells. Patient A, anti-Tdt antibody, BA-HRP cytospin marrow. (G) Note the consistent cytoplasmic μ expression found in long-term culture B cells. Culture B, anti-μ antibody, BA-HRP, cytospin preparations. (H) Note the nuclear proliferating antigen (Ki-67) found in the majority of culture B cells. Black nuclear coloration indicates a proliferating cell, and the blue counterstain reveals a minority of resting cells. Culture B, anti-Ki67, BA-HRP, cytospin preparations.**



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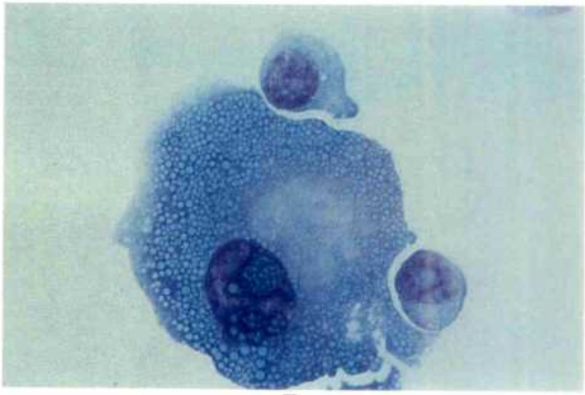

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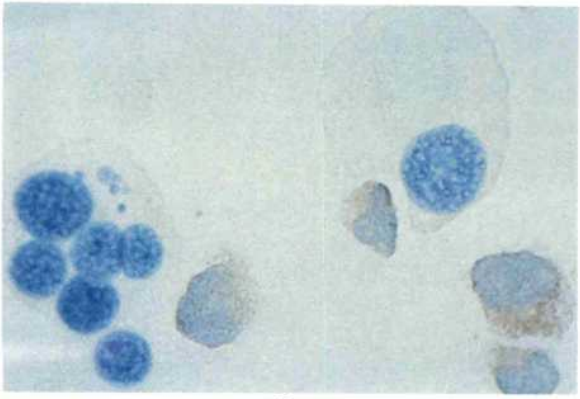

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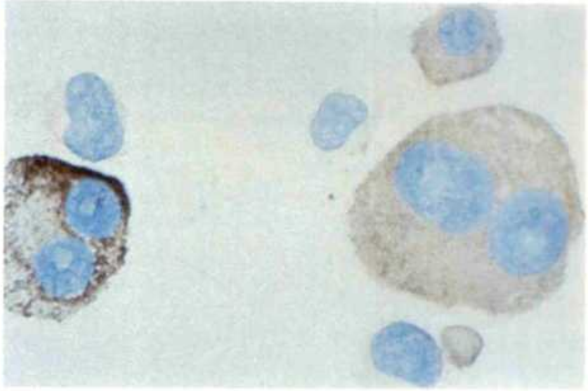

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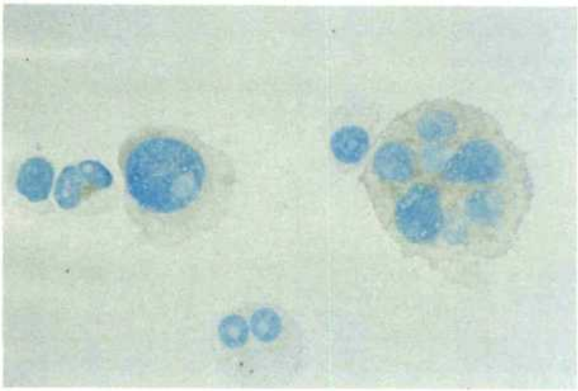

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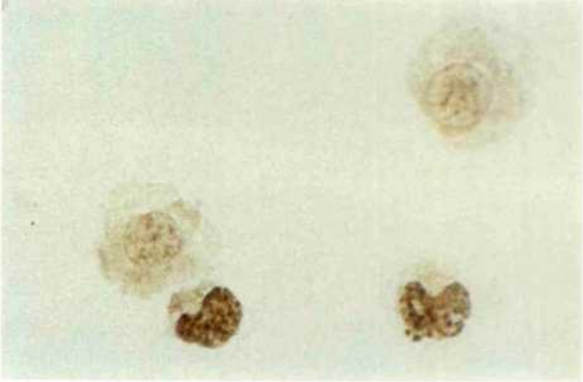

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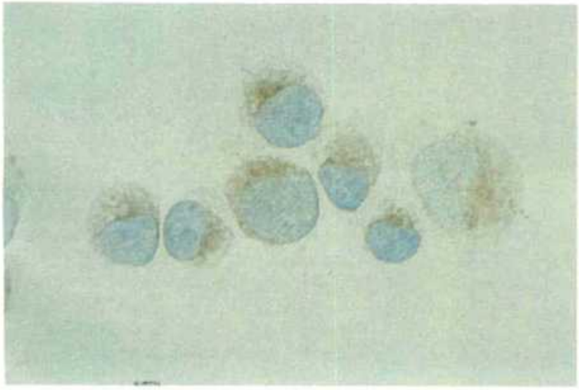

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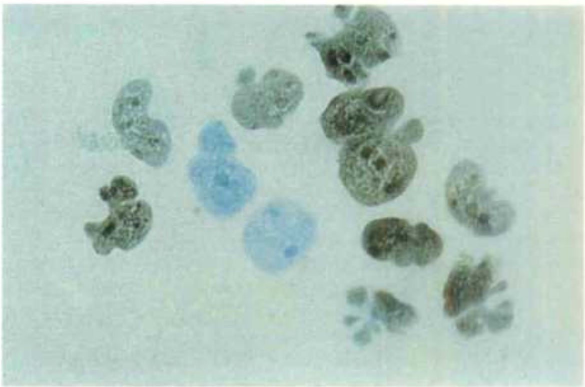

H

by O'Farrell.[23,24] The mol wt standards obtained from Bio-Rad consisted of the following components: lysozyme, 14,400 mol wt; soybean trypsin inhibitor, 21,500; carbonic anhydrase, 31,000; ovalbumin, 45,000; BSA, 66,200; phosphorylase B, 92,500; and β-galactosidase, 116,250.

Labeling Index and Proliferative Index

The labeling index (LI) was performed by using previously described one-hour "flash labeling" with [^{3}H]thymidine.[29] The proliferative index represented the percentage of cells reacting with the monoclonal antibody Ki-67 (DAKO, Dakopatts, Copenhagen).[30,31] Ki-67 identifies cells throughout the cell growth cycle and not resting cells.[30,31]

Stimulation Studies With Phorbol Ester

Culture C was stimulated with phorbol ester 12-0-tetradecanoyl-phorbol-13-acetate (TPA) by using the methods and controls described by Caligaris-Cappio et al.[11] The TPA was dissolved first in acetone and then absolute alcohol and used in the cultures at a concentration of 1.6×10^{-5} mol/L. Control and TPA-induced cells were harvested after 72 hours, assessed for viability, and then phenotyped by using biotin–avidin–horseradish peroxidase (BA-HRP) methods as detailed earlier.

Immunoglobulin Gene Rearrangement Analysis

High–mol wt DNA was extracted from a frozen aliquot of myeloma cell culture C by using quanidium isothiocyanate buffer and standard methods.[32] DNA was digested with *Hind*III or *Bam*H1, size-fractionated on 0.6% agarose gels, and transferred by Southern blotting to nitrocellulose. Cloned genomic DNA fragments containing the immunoglobulin heavy-chain joining region (J_H) or κ light-chain constant region (C_κ), kindly provided by Dr Philip Leder,[33,34] were labeled with dCTP (deoxycytidine triphosphate) [α-^{32}P] by nick translation to an activity of at least 10^8 cpm/μg. After hybridization with the probes the filters were washed under conditions of high stringency, and autoradiography was performed.

RESULTS

Morphology

As revealed in Fig 1A and B, the Wright-stained marrow aspirate of patient A revealed cells with typical and atypical plasma cell morphology as well as immature lymphoid cells. The typical plasma cells showed abundant cytoplasm with a prominent hof region and a single eccentric nucleus with occasional nucleoli. Some also revealed cytoplasmic vacuolation (Mott cell formation) and others, nuclear inclusions (Dutcher body formation). Both Mott cells and Dutcher bodies persisted in initial culture. Atypical plasma cells were multinucleate, sometimes with "cloverleaf nuclei" with blastic-looking chromatin and prominent nucleoli. The admixed immature lymphoid cells occurred in both direct preparations and subsequent cultures, ultimately becoming the dominant feature in long-term culture. The lymphoid cells were generally smaller than plasma cells with eccentric nuclei and prominent nucleoli (Figs 1A to F). Frequently, the immature lymphoid cells appeared nestled within lacunae of large plasma cells (Figs 1A, B, and C). In the other two culture lines (cultures B and C), a few typical plasma cells could be found in one (culture B), whereas the other (culture C) was largely devoid of typical plasma cells. Both myeloma cultures were comprised largely of small blastic lymphoid cells (Fig 1G).

Immunotyping Studies

There was general agreement between flow cytometry and cytospin immunotyping in both direct and cell culture tissues, with pre-B phenotypes readily detected by both. Cytospin typing allowed simultaneous delineation of morphology and immunotype (Figs 1C to H); flow cytometry allowed delineation of coexpression of antigens as revealed by coplots (see Figs 2 and 3). Both allowed delineation of surface *v* cytoplasmic antigens critical to pre-B cell definition.

By cytospin immunoperoxidase determination, patient A initially had a complex marrow phenotype (see Fig 1C through F and Table 1) with the large plasma cells expressing γ heavy chain, κ light chain, CALLA, B1, TRF, Leu 12, Leu 14, Ia, PCA-1, and PC-1, which indicates a monoclonal immunoglobulin-producing plasma cell proliferation with some immature B antigens (Fig 1E).

The smaller lymphoid cells in patient A expressed μ heavy-chain, CALLA, Ia, TRF, pan-B antigens (B1, B4, Leu 12, Leu 14), and PCA-1 and PC-1. The expression of PCA-1 and PC-1 was fainter in smaller lymphoid cells than in larger plasma cells. Tdt was strongly expressed in a nuclear pattern in the smaller lymphoid cells and notably on a few atypical-appearing plasma cells (Fig 1F).

The large plasma cells in patient A differed from the small lymphoid cells by immunoglobulin isotype (γ *v* μ) but had in common the apparent coexpression of PC-1, PCA-1, CALLA, B1, Leu 14, Leu 12, Ia, and TRF. Most lymphoid cells expressed nuclear Tdt, as did some plasma cells, thereby indicating some overlap with this enzyme as well.

The pre-B phenotype described in the smaller lymphoid

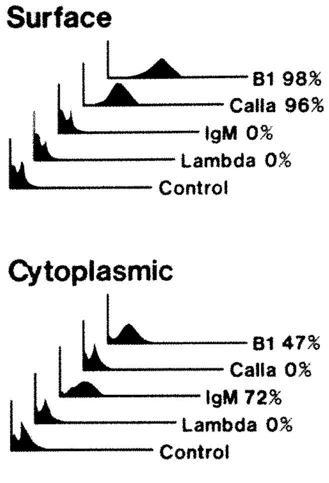

Fig 2. Flow cytometry assessment of myeloma culture C indicating pre-B cell phenotype: CIgM+, SIg−. Coplots of surface antigens reveal coexpression of B surface B1 and CALLA with absent IgM, λ and κ (not shown), whereas cytoplasmic antigens determined after swelling buffer and fixation reveal coexpression of IgM and B1 with an absence of CALLA, λ and κ (latter not shown). Additional study also revealed coexpression of PC-1 (not shown), which indicates a pre-B/plasma cell hybrid.

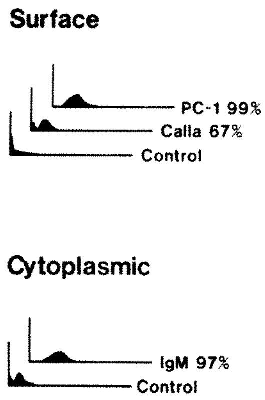

Fig 3. Flow cytometry assessment of myeloma culture A revealing surface coexpression of PC-1 and CALLA and cytoplasmic expression of μ, which indicates a hybrid phenotype of pre-B cell and plasma cell.

cells—CIgM+, SIg−, B1+, B4+, Leu 14+, Leu 12+, CALLA+, Tdt+, PCA-1+, PC-1+—suggests a novel association between the pre-B phenotype and plasmacytic antigens (PCA-1, PC-1). The expression of CALLA, some Tdt, B1, Leu 14, and Leu 12 by the plasma cells indicates an element of immaturity among the plasma cell component as well. In summary, the immature B cell component has mature plasma cell features, and the mature plasma cell has pre-B features; each appears then to be a hodgepodge or hybrid of B cell phenotypes, which suggests first, aberrancy in neoplasia and, second, linkage between the two.

In the short-term cultures of patient A these precise phenotypes in both the large and smaller cells persisted. In longer-term culture (culture A), the smaller lymphoid cells with the following pure phenotype emerged and became exclusive: CIgM+, SIg−, B1+, B4+, Leu 12+, Leu 14+, CALLA+, Tdt+, PCA-1+, PC-1+.

Culture B yielded a nearly pure novel pre-B phenotype: CIgM+, SIg−, B1+, Leu 12+, Leu 14+, PCA-1+, PC-1+, IL-2+, Tdt+, Ia+, and TRF+ (Table 1). Culture C was quite similar except for variable PCA-1 staining. The initial faint PCA-1 staining was lost in long-term culture, but PCA-1 expression returned with TPA induction as indicated in Table 1. The TPA-induced cells also lost CALLA expression coupled with increased PCA-1 expression. Thus, a slight tendency for pre-B–like cell maturation is indicated.

Extensive flow cytometry assessment of myeloma cultures A and C, as revealed in Figs 2 and 3, confirmed cytoplasmic μ expression by direct IF on fixed cells, surface immunoglobulin absence by indirect IF on viable unfixed cells, and coexpression of surface B1, CALLA, and PC-1 in these cells. Demonstration of cytoplasmic μ in these cells required the use of the aforementioned swelling buffer and the use of the direct IF method of detection. The cytometry immunophenotype fully mirrored the cytospin immunoperoxidase immunotype except that flow cytometry detected faint B2 expression in some cells from culture C.

Kinetic Studies

Studies of proliferation in two myeloma culture lines were made by using both monoclonal antibodies and the tritiated thymidine LI. Ki-67 is a mouse monoclonal antibody recognizing a human nuclear antigen present in proliferating cells but absent in resting cells.[30,31] This monoclonal reacted with 40% to 50% of the myeloma cells in cultures A and C. As indicated in Fig 1H, this antigen was strictly nuclear in location. After initial experiments established the high percentage of Ki-67 positivity in nuclei, we used double-labeling experiments using a mouse anti-μ monoclonal admixed with a mouse anti–Ki-67 as a monoclonal cocktail. These experiments successfully detailed cytoplasmic reactivity of μ and nuclear reactivity of anti–Ki-67, thereby indicating that the Ki-67 proliferating cell is a CIgM+ pre-B–like cell representing a major growth fraction of these myeloma neoplasms.

This Ki-67 proliferative index of 40% to 50% for myeloma cell line C correlates with the thymidine LI of 36% to 37% on the same culture material. Thus, by both the Ki-67 proliferative index and the [^{3}H]thymidine LI the pre-B cell cultures had a striking proliferative component greatly exceeding that commonly found in myeloma.

Interestingly, double labeling with Ki-67 and μ revealed some μ-positive cells that were Ki-67−, thereby indicating that not all myeloma pre-B cells are proliferative; some are resting.

We also note with interest that myeloma line C had an LI of 1% at the time of initial diagnostic marrow. The transition from an initial 1% LI to a subsequent 36% to 37% LI reflects a rapid growth transition, probably related to the emergence of the pre-B–like cell component in culture.

Characterization of Cytoplasmic μ in pre-B Myeloma Cells Using 1- and 2-D Gel Electrophoresis and Immunoblotting

Cytoplasmic μ immunoglobulin as a stage-specific protein marker of B cell immaturity was the object of immunochemical, structural study. Because myeloma cell line C was a nearly pure proliferation of pre-B myeloma cells, this was chosen for detailed study.

As revealed in Fig 4, SDS–gel electrophoresis in one dimension allowed the separation of numerous pre-B myeloma proteins by size. Immunoblots of the same separation revealed a single discreet μ band and absent light chains (λ, not shown). In comparison, standard IgM had a broader μ band and a clear demonstration of light chain (κ shown). A plot of the logarithm of mol wt *v* migration ratio from mol wt standards revealed a mol wt of 74 kilodaltons (kDa) (range, ±2,000) for the pre-B myeloma μ, as well as standard μ.

Two-dimensional gel electrophoresis coupled with immunoblotting provides another facet to the characterization of this B cell differentiation protein: separation by charge as well as size. As revealed in Fig 5A, this 2-D gel fingerprinting was also performed on myeloma cell line C and revealed a complex fingerprint of proteins. Immunoblotting of this fingerprint (Fig 5B) reveals that μ in this pre-B myeloma cell line is a single discrete protein demonstrating homogeneity in size and charge. This discreteness was in sharp contrast with 2-D gels of standard IgM, which showed considerable size and charge heterogeneity. The discreteness of pre-B μ is believed due to its monoclonal origin and thus speaks for neoplastic aberrancy.

Figure 6 combines, in a single 2-D gel, mol wt and μ

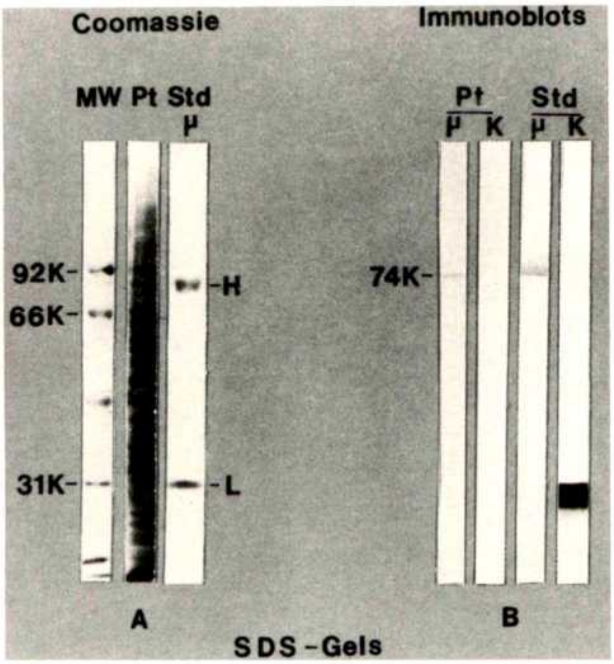

Fig 4. Mol wt determination of cytoplasmic μ from culture C myeloma cells. (A) Comparison of 1-D SDS gels including mol wt standard, patient (pt) C culture cells, and μ standard. Note that the nonspecific Coomassie stain identifies the broad range of culture C proteins and the specific heavy (H) and light (L) chain bands in the standard μ. (B) After Western blotting/immunoblotting the anti-μ antibody detects a single discrete 74 kDa μ band in patient C (Pt) and a broader standard (Std) μ 74 kDa band. The anti-κ and λ (not shown) were absent in the pre-B cell culture and present in the standard.

standards and pH measurements to facilitate determination of the pI. Given the discreteness of the pre-B μ, its pI was estimated as described by O'Farrell to be approximately pH 6.3 ± 0.2 units.

Genotype of Myeloma pre-B Cells (Line C)

As revealed in Fig 7, myeloma culture C shows two heavy chain (J_H) gene rearrangements compared with the germline control. As illustrated, increasing amounts of DNA were analyzed, with no germline band detected. In Fig 8 the same culture shows one κ (C_κ) light-chain gene rearrangement and one germline band, thereby indicating that one κ allele is rearranged and the other is in the germline state. Collectively, these results indicate that line C is a monoclonal population of B cells.

DISCUSSION

In this study the coexpression of immature B cell and plasma cell antigens in cells from myeloma bone marrows is investigated in detail. We delineate the previously unrecognized occurrence of a novel pre-B–like cell component in plasma cell myeloma and describe the emergence of this component in long-term culture. Material from three myeloma patients was studied. In one (patient A), the initial marrow was found to have two unusual admixed cell populations: (*a*) numerous immature lymphocytes with a phenotype normally found in pre-B cells, but with the addition of plasma cell antigens (eg, PC-1); and (*b*) many plasma cells with immature B antigens (eg, CALLA). This rare occurrence has been termed CALLA-positive myeloma and is associated with a poor prognosis.[10-12] The coexpression of plasmacytic and immature pre-B antigens within these neo-

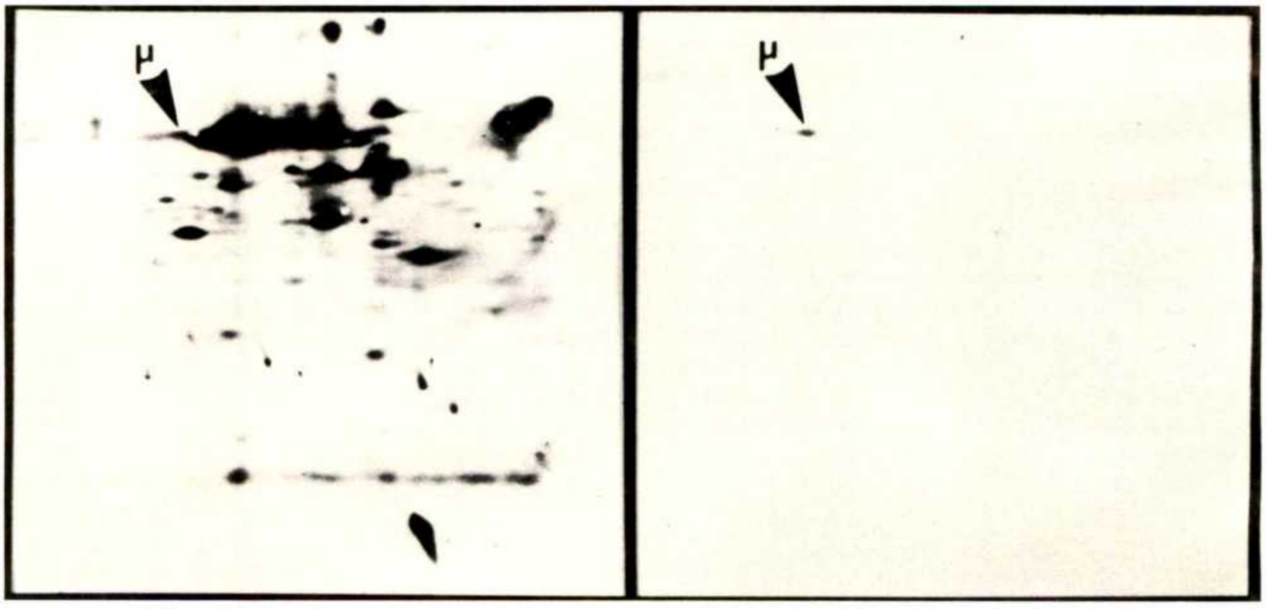

Fig 5. Two-dimensional gel characterization of pre-B–like μ in culture C myeloma cells. (Left) Note the complex fingerprint of proteins stained with Coomassie blue. (Right) Immunoblotting of this fingerprint with an anti-μ antibody reveals that μ in this pre-B–like myeloma cell line is a single discrete protein demonstrating homogeneity in size and charge.

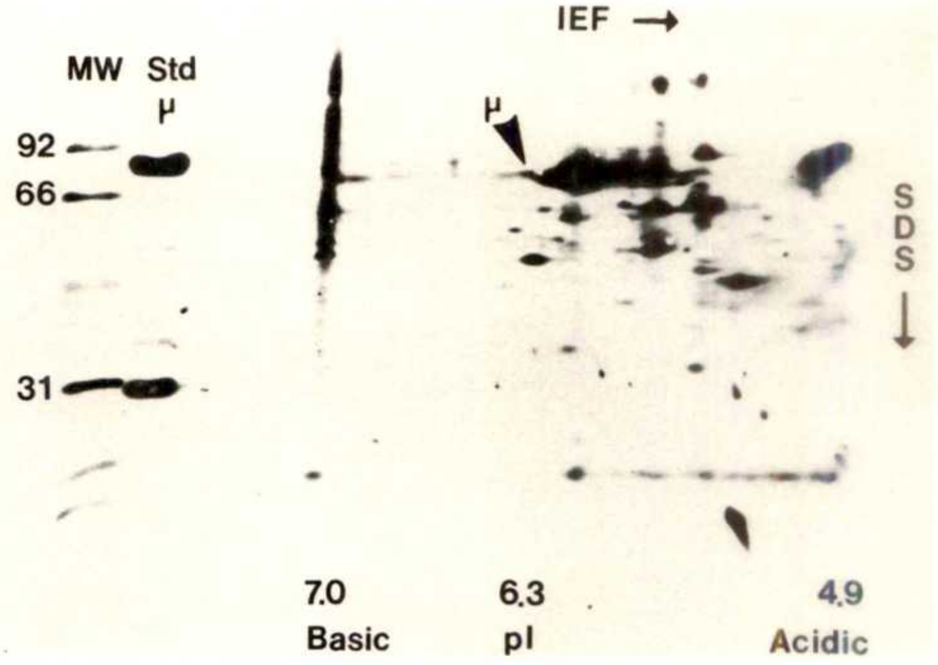

Fig 6. Combined 2-D gel, controls, and standards to reveal the μ isoelectric point (pI). pH determinations of the IEP rods revealed a range from 4.9 to 7.0, with the μ pI at 6.3 ± 0.2 units.

plastic lymphoid cells constitutes a novel phenotype that persisted in subsequent culture. In the other two cultures (B and C), the lymphocytes with coexpression of plasma cell and pre-B antigens constituted a distinctly rare subpopulation (<1%) in the initial direct marrow material, and they were admixed with myeloma plasma cells of mature phenotype without CALLA or other immature B antigen expression. Both of these patients had stable disease without an immature CALLA+ myeloma phenotype. These novel pre-B–like cells emerged as a dominant feature only in subsequent long-term culture. In all three patients the availability of large numbers of the novel pre-B–like cells from M-3 cultures allowed the various detailed studies to be performed: documenting cells with (*a*) high proliferative capacity (Ki-67+, high LI percentage), (*b*) aberrant μ expression, (*c*) monoclonality as evidenced by immunoglobulin gene rearrangement analysis, and (*d*) some capacity to mature on exposure to TPA.

The emergence of the pre-B–like cell component in culture in our cases most probably reflects their high proliferative index. These highly proliferative pre-B–like cells could represent a significant aspect of the self-renewal capacity of myeloma.[30,31,35-38] Specifically, the pre-B–like component could represent the stem cell population of myeloma.[36] Previous evidence noting pre-B cells in myeloma emphasized common idiotypic antibody expression in both pre-B cells and plasma cells that led to a view that the clonal origin of myeloma may begin with normal precursor pre-B cells.[8] It has been assumed that the malignant plasma cell or myeloma cells with features in common with very immature normal B lymphocytes stem from that particular phase of normal B lymphocyte development.[8,36] However, our data suggest the contrary, that these malignant cells have novel phenotypes, a hybrid of immature B and plasma cell antigens, that are not part of the normal B cell expression of phenotypes. They may simply represent myeloma cells with aberrant or disorganized gene expression that produce novel phenotypes.[10,36] Descriptions of CALLA expression in myeloma plasma cells have also led to this speculation.[10,11]

This novel association between immature B antigens (μ, CALLA, Tdt) and plasma cell antigens has not previously been described or suspected in normal B cell development.[1,2] In normal B cell differentiation, PCA-1 and PC-1 are expressed only at the terminal step (the plasma cell).[1,2] PC-1 and PCA-1 have been consistently noted in plasma cell leukemia, myeloma, plasmacytomas, and Waldenström's macroglobulinemia but not on immature B cell neoplasms like acute lymphocytic leukemia.[1,2] Normal plasma cells possessing PC-1 and PCA-1 expression are generally noted to lack B1, B2, Ia, Tdt, and CALLA.[1,2] Pre-B cells are specifically said to be positive for cytoplasmic μ, B1, CALLA, Tdt, and Ia[3,4] and negative for surface Ig, PCA-1, and PC-1.[1,2,35] The terminal plasma cell differentiation associated with plasma cell antigens is specifically initiated through contact with antigens[1,2,35]; therefore, the pre-B–like cells with PC-1 and PCA-1 expression we describe probably derive from mature antigen-experienced cells, not the usual antigen-independent origin of normal pre-B cells.[37] Our findings question the view that malignant myeloma cells necessarily express antigens in the normal sequential fashion found in normal B cell differentiation. Nonetheless, our TPA findings and others[11] indicate that progression from immature B-like cells to more mature plasmalike cells (eg, loss of CALLA, increase of PCA-1 expression) may sometimes be chemically induced.

The key development in this study was the emergence of the sustained pre-B cell growth in culture using the recently devised M-3 culture medium.[13] A major obstacle in the

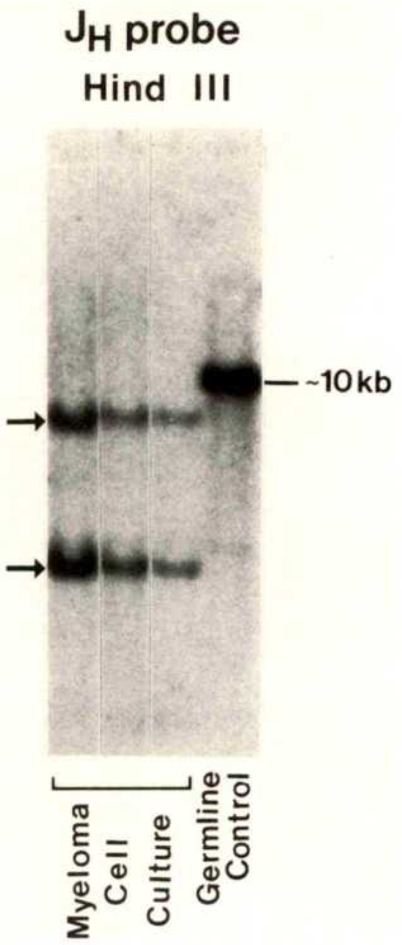

Fig 7. Myeloma cell culture from patient C shows two heavy-chain gene rearrangements (arrows, left three lanes) compared with the germline control (bar, right lane). Increasing amounts of myeloma cell culture DNA were analyzed in an attempt to detect a faint germline band, but no germline band was detected (even though the left lane contains 1.5 times the amount of DNA used in the right-hand, germline control lane). These results indicate that the myeloma cell culture studied is monoclonal, that both heavy-chain alleles are rearranged using J_H probe, and that germline DNA from contaminating nonclonal cells is absent (within the 1% to 5% sensitivity of this method).

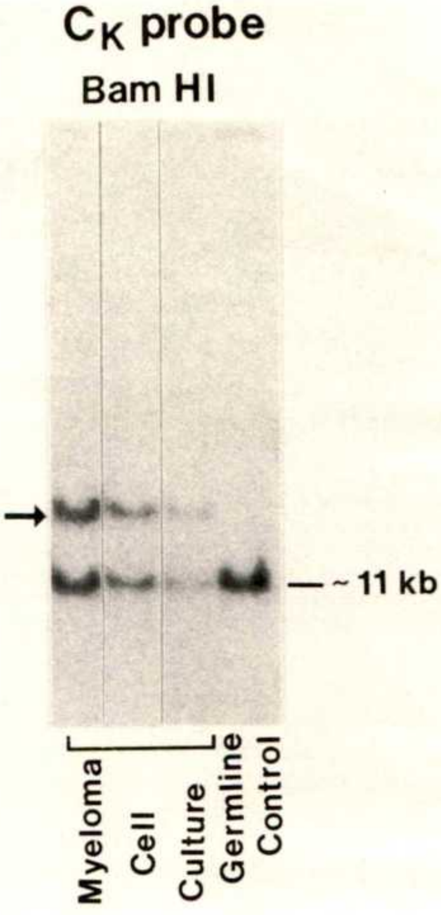

Fig 8. Myeloma cell culture from patient C shows one κ light-chain gene rearrangement (arrow, left three lanes) and one germline band compared with the germline control (bar, right lane). Increasing amounts of myeloma cell culture DNA were analyzed in an attempt to detect other, faint rearranged bands, but none were found. These results indicate that the myeloma cell culture studied is a monoclonal population of B cells and that one κ allele is rearranged using C_κ probe and one allele is in the germline state.

study of human B cells has been the general lack of success with long-term cultures of immature B cells.[35,39] In the past, cell lines developed from normal peripheral blood have most commonly been mature polyclonal SIg-bearing lymphoblastic lines.[37] To date, the rare human pre-B cell lines have derived from one of two pathological states: (*a*) X-linked agammaglobulinemia (XLAG)[39] and/or (*b*) acute lymphocytic leukemia.[40,41] We may now add myeloma to this list. It is speculated that the key to pre-B line emergence in XLAG may be the lack of inhibition by mature B cells.[39] Similarly, the exceptional and ready emergence of a pre-B–like line in our patient A may relate to the aberrancy of plasma cells (eg, CALLA+, Tdt+ phenotype). The CALLA+ plasma cell component in patient A might have been permissive to pre-B cell growth in vivo. However, because patients B and C did not have conspicuous CALLA+ plasma cells, this clearly could not be the only variable allowing emergence and growth of the pre-B–like cells. Perhaps the lack of associated idiotype-specific regulatory T cells in vitro was pivotal.

Although there are few human pre-B lines, mouse pre-B cell lines are numerous.[14,35,38,42-46] The murine pre-B lines reveal some findings relevant to our study of human myeloma pre-B cells. For example, murine pre-B cell lines are specifically proved to lack plasma cell antigens,[35] in contrast to our novel human myeloma pre-B cell lines. Furthermore, murine pre-B lines demonstrate Ig gene rearrangements as found in our line C, which indicates that pre-B cells are a key step in the production of antibody diversity.[35,44,45] Murine pre-B cell lines may progress to secretion of IgM and plasma cell formation with concanavalin A[35] and other stimuli.[44,45] Murine pre-B lines have also served as a homogeneous source of pre-B cells allowing structural analysis of μ chains.[14,42] Clearly, pre-B lines offer all of the prospects of genotypic, phenotypic, and stimulatory studies and structural analysis. With plans to pursue all of these avenues further, we have focused presently on the structural analysis of μ as the unique stage-specific protein of pre-B cells.

Our electrophoresis and immunoblotting studies reveal the unique, aberrant nature of the μ chains found in myeloma pre-B–like cells. Immunoblots of SDS-PAGE separations reveal a pre-B–like μ band that migrates with a mobility similar to the standard secretory μ chain in the range of 74 kDa under reducing conditions. The same immunoblots also reveal differences. In our pre-B–like cells, absent light chains and a single discrete μ contrast with a broader μ band and definite light chains expressed by standard μ secretory cells. Two-dimensional gel electrophoresis immunoblotting also revealed that myeloma pre-B–like μ is a single discrete protein, homogeneous in size and charge (mol wt 74 kDa; pI – 6.3). This discreteness was in contrast to the 2-D gels of standard μ that showed considerable size and charge heterogeneity due to its polyclonal origin. The discreteness of myeloma pre-B–like μ is believed due to its monoclonal origin and bespeaks neoplastic aberrancy. Our mol wt and pI determinations represent the first 2-D gel characterization of a human pre-B–like μ; previous characterizations have been of animal pre-B μ.[14]

The discreteness of the "malignant" μ in our pre-B–like cells contrasts with the heterogeneity of pre-B μ described in murine fetal liver pre-B cell hybridomas.[14] In the murine model the different mol wts of pre-B μ chains derive from different hybridomas and are related to minor peptide differences reflecting polyclonal origin.[14] Normal human serum also reveals heterogeneity of μ chain expression, again thought to reflect polyclonal peptide differences.[27] Surprisingly, secreted monoclonal heavy chains from patients with monoclonal gammopathies and plasma cell dyscrasias usually demonstrate size and charge heterogeneity, not discrete spots as expected in malignancy. This heterogeneity, in spite of monoclonality, is thought to reflect variable glycosylation or degradation products.[27] The homogeneity of our pre-B–like μ contrasts with all three circumstances, thus reflecting its structural simplicity. A single myeloma pre-B–like μ spot speaks for a single monoclonal source of a mutant μ.[23] This discreteness also favorably indicates no artifactual charge heterogeneity due to faulty technique.[23]

The monoclonal nature of these myeloma pre-B lines is further evidenced by the finding of heavy- and light-chain immunoglobulin gene rearrangement when using J_H and C_K probes in pre-B line C. It should be noted that serial passage of the pre-B lines was not done by a limiting-dilution technique, so these serial passages represent the progeny of multiple tumor cells rather than a single isolated tumor cell. Thus, the phenotypic and genotypic evidence of monoclonality described earlier cannot be ascribed to the artifact of studying the progeny of a single tumor cell. These preliminary phenotypic and genotypic studies do not precisely define the relationship of the pre-B lines to the original neoplastic stem cell or to the original neoplastic plasma cells. However, these techniques applied prospectively and in a comparative fashion to the original myeloma cells and to multiple discrete pre-B clones derived from these cells hold the promise of defining these relationships and thus the nature and dynamic of self-renewal in myeloma. Although major in scope, this type of study is justified by the results described in this paper and made feasible by the ability to culture pre-B–like cells from human myelomas.

Although we have identified several characteristic features of novel pre-B cells as myelomalike, the origin and significance of these cells remain unclear. There are, for example, unanswered questions about the relationship of the pre-B–like component to the plasma cell component. The mechanism facilitating the emergence of pre-B–like cells in vivo, which carry such a poor prognostic significance, remains unknown. The reasons for in vitro outgrowth of pre-B–like cells in more usual myeloma also require clarification. Nonetheless, these phenomena are of major clinical significance. Three studies now associate CALLA+ myeloma plasma cells with lethal progressive myeloma.[10-12] Beyond prognostic use, perhaps the most valuable outcome of this study was the creation of stable, long-term pre-B–like myeloma cell cultures. These cultures present a major resource for scientific study. Myeloma pre-B–like cell cultures offer a stable source of cells to study disordered and evolving B cell differentiation and proliferation; they allow the study of immature B cells, which may be critical to the immortalization of myeloma.

Karyotypic analysis of initial direct marrow material and later pre-B cell cultures from patient B indicates the presence of a marker chromosome $(7q^-)$ in both samples, substantiating the clonal origin of the pre-B culture in this instance.

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Therapy of Chronic Myelogenous Leukemia With Recombinant Interferon-γ

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Recently, we reported that recombinant interferon-α (rIFN-α) can induce hematologic remissions and cytogenetic improvement in newly diagnosed Philadelphia (Ph)-positive chronic myelogenous leukemia (CML) patients. Although IFN-γ is a structurally distinct molecule, this agent suppresses in vitro hematopoietic progenitor cells in a fashion similar to that of IFN-α. Therefore, we initiated a study of rIFN-γ at doses of 0.25 to 0.5 mg/m^2/d intramuscularly in patients with Ph-positive benign-phase CML. Twenty-six of 30 patients entered in the study were evaluable. Six patients have achieved a complete hematologic response; four, a partial hematologic response. The median follow-up period of patients who are in complete remission is 7.5 months (range, 5 to 12 months). No relapses have occurred among the complete responders. So far, five patients have had cytogenetic improvement with emergence of 5% to 45% diploid cells in the bone marrow. Fever and flulike symptoms were the most common side effects, with partial tolerance often developing after about 1 week. The majority of patients tolerated therapy with minimal change in performance status. In conclusion, rIFN-γ has demonstrated clinical activity in CML. On the basis of these observations and the in vitro synergistic growth-inhibitory effects of IFN-α and IFN-γ, we have started trials of combination IFN therapy in CML patients.

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INTERFERONS (IFN) ARE A GROUP of naturally occurring proteins with potent antiviral, antiproliferative, and immunoregulatory activities that have stimulated interest in their evaluation in human malignant, infectious, and immunodeficiency diseases.[1-3] On the basis of their antigenic and physiochemical properties, the IFNs are divided into three classes: IFN-α (leukocyte), IFN-β (fibroblast), and IFN-γ (immune). IFN-α is a family of at least 14 highly homologous species produced by leukocytes. IFN-β is produced by fibroblasts and epithelial cells. IFN-α is structurally related to IFN-β, and both are virally induced, acid-stable molecules. In contrast, IFN-γ is a structurally distinct, acid-labile glycoprotein produced primarily by T lymphocytes in response to antigen or mitogen.[4]

α-IFNs have been demonstrated to have significant antitumor activity in hairy cell leukemia and other tumors.[5-7] A large body of evidence demonstrates significant anticellular effects of IFN-α on normal myeloid stem cells as well as chronic myelogenous leukemia (CML) progenitor cells in vitro.[8-10] In addition, we have recently reported that recombinant IFN-α (rIFN-α) can induce hematological remissions and, more importantly, cytogenetic improvement in Philadelphia (Ph) chromosome–positive CML patients.[11,12] These results were of special interest because the invariably fatal course of CML has not been significantly altered by chemotherapeutic agents that control the benign phase of the disease but do not affect the Ph chromosome. In vitro, IFN-γ suppresses myeloid colony formation in a manner similar to IFN-α[13,14] and can induce differentiation of leukemic cells.[15] Synergistic growth inhibitory effects are observed when IFN-γ and IFN-α are combined[13]; this may be related to their binding to distinct human cell surface receptors.[16] These synergistic properties may eventually be exploitable in the treatment of leukemia. Therefore, we initiated an investigation to ascertain the tolerance and efficacy of IFN-γ in benign-phase CML patients.

MATERIALS AND METHODS

IFN. The isolation, manufacture, characterization and purification of human rIFN-γ were performed by Genentech, Inc. The gene for human IFN-γ was cloned and expressed in *Escherichia coli* by methods previously described.[17,18] Replication of the IFN gene occurs as the *E coli* bacteria divide, thus permitting the production of large amounts of rIFN-γ. The material used in this investigation is identical in sequence to native human IFN-γ except for the presence of a methionine residue at the *N*-terminal.[19] The final purity of rIFN-γ was 98% as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The direct inoculation technique was used to confirm sterility. The final product contains <0.5 ng endotoxin/mg protein as determined by the limulus amebocyte lysate assay. The specific activity of rIFN-γ was approximately 2×10^7 U/mg protein based on antiviral activity assessed by inhibition of encephalomyocarditis virus replication in A549 cells (human lung carcinoma cell line), corrected to the reference IFN standard Gg 23-901-530 of the National Institutes of Health, Bethesda, MD.

The IFN was provided in the form of a sterile lyophilized powder. Immediately before administration, the powder was reconstituted with sterile water to a concentration of 1.0 mg/mL.

Patient selection. CML patients whose disease was in the benign phase were entered in the study. Patients were considered to be in benign phase if blasts constituted less than 15% of bone marrow cells, their disease was responsive to hydroxyurea, and in patients whose disease had been diagnosed more than 1 year before the study, cytogenetic analysis showed no evidence of clonal evolution. Eligibility criteria were as follows: Karnofsky performance status $\geq$50%,[20] no antileukemia treatment for at least 2 weeks before initiation of IFN treatment, evidence of relapsing leukemia reflected by increased WBC counts to levels $\geq 20 \times 10^3/\mu L$, life expectancy of at

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least 3 months, and preserved hepatic (bilirubin ≤1.5 mg/dL) and renal function (creatinine ≤2 mg/dL). Patients requiring ongoing therapy with corticosteroids or nonsteroidal antiinflammatory drugs were excluded from the study. Informed consent was obtained from all patients according to institutional policy.

Treatment plan. The patients were followed in the outpatient clinic of The University of Texas M.D. Anderson Hospital and Tumor Institute at Houston for the first 2 weeks and then continued their treatment at home. The treatment regimen was chosen on the basis of our experience in phase I studies, which showed that rIFN-γ is absorbed after intramuscular (IM) administration[21] and demonstrates many biologic properties in vivo including activation of macrophages.[22] The maximum tolerated dose determined in our phase I trial[23] was used in this study. Patients received 0.25 $mg/m^2/d$ of IM administered rIFN-γ for seven days. If this dose was tolerated with a ≤20% decrease in Karnofsky performance status, the dose was increased to 0.5 $mg/m^2/d$ thereafter. This dose was reduced by 50% in case of any of the following toxicities: a ≥30% decline in Karnofsky performance status, an increase in serum liver enzymes five or more times the normal values, or an increase in the serum creatinine level to ≥2.5 mg/dL. A further 50% dose reduction was incorporated if toxicities continued. The induction phase of the study lasted 6 weeks. At the end of induction, all patients with evidence of complete or partial hematologic remission, or hematologic improvement, were maintained on the same dose and schedule of rIFN-γ.

Each patient had a physical examination before the study, and the size of the spleen and liver was assessed. Complete blood cell counts, differential, and platelet counts were performed twice in the four days preceding the first injection. Pretreatment assessment included 12-channel blood chemical analysis, serum electrolyte analysis, determination of serum lactate dehydrogenase level, anti–rIFN-γ antibody assay, leukocyte alkaline phosphatase score, urinalysis, ECG, and technecium 99 scan of the liver and spleen. Bone marrow biopsy and aspiration samples were evaluated for cellularity, morphology, and cytogenetic analysis. The cytogenetic techniques used on the bone marrow have been previously described,[24] and ≥20 metaphases were counted.

During the course of the study, the CBCs were repeated two to three times weekly during the first month and two times per month thereafter. Bone marrow aspiration, biopsy and cytogenetics, and liver/spleen scan were repeated every 2 to 3 months.

Criteria for response. A complete hematologic remission required normalization of the peripheral WBC counts to levels of less than $9 \times 10^3/\mu L$ with normal differential counts and no immature forms (blasts, promyelocytes, myelocytes, or metamyelocytes) and the disappearance of all clinical symptoms and signs of disease including palpable splenomegaly. A partial hematologic remission was defined as a decrease in the WBC counts to at least 50% of pretreatment levels and to less than $20 \times 10^3/\mu L$. Patients whose peripheral WBC counts had normalized but who had persistent splenomegaly or immature peripheral cells were included in this category. Treatment was considered to have failed for all patients who had less than a partial hematologic remission.

IFN antibody assays. The presence of antibodies to rIFN-γ was determined on blood samples obtained before and after completion of the study as well as every month while the patients received IFN. Testing for antibodies was performed by a radioimmunoprecipitation assay. The patient's serum was incubated with $[^{125}I]$-labeled rIFN-γ, which was followed by immunoprecipitation with goat antihuman IgG. The radioimmunoprecipitation assay is capable of detecting neutralizing antibody and is approximately ten- to 100-fold more sensitive than the neutralization assay for equivalent epitopes (Genentech, data on file).

RESULTS

Patient characteristics. Thirty CML patients (19 men and 11 women) were entered in the study. Four patients were considered inevaluable for response because of a major protocol violation (two patients), noncompliance (one patient), and toxicity requiring discontinuation of therapy on day 10 (one patient). The median age of the evaluable patients was 47 years (range, 22 to 66 years). All patients were in the benign phase of the disease. Their median time from diagnosis was 9 months (range, 1 to 60 months). Twenty of the evaluable patients had prior chemotherapy, usually with hydroxyurea and/or busulphan.

The karyotype of patients was as follows: 23 patients, t(9;22) in 100% of bone marrow metaphases; one patient, t(4;9;22); one patient, t(8;17)t(9;22) in 90% of metaphases and t(9;22) in the remaining 10% of metaphases; and one patient, diploid. On Southern blot analysis, the latter patient had the rearrangement in the breakpoint cluster region (*bcr*) on chromosome 22 characteristic of Ph-positive CML. Such patients have previously been shown to have a clinical course indistinguishable from that of patients in whom the Ph chromosome can be demonstrated by cytogenetic analysis.[25]

Response. Six patients achieved a complete hematologic remission; four, a partial hematologic remission. Pre- and posttherapy hematologic parameters of these patients are shown in Table 1. Complete responders showed a normalization of WBC and platelet counts and the disappearance of immature cells from the peripheral blood. Bone marrow changes included a decrease in the cellularity from a median baseline value of 100% (range, 90% to 100%) to a posttherapy value of 75% (range, 50% to 100%). The bone marrow blasts decreased from a baseline value of 10% (range, 2.6% to 14.6%) to a posttherapy value of 3.2% (range, 1.2% to 5.0%). In the patients failing rIFN-γ treatment, therapy was generally discontinued because of rising or unresponsive WBC counts; no patient developed blast crisis while on study. The median interval to complete hematologic remission was 1 month (range, 0.5 to 5 months). The median follow-up period of patients who achieved a complete hematologic remission is 7.5 months (range, 5 to 12 months). There have been no relapses among the complete responders. To date, five of six

Table 1. Changes in Hematologic Parameters Among Ph-Positive CML Patients Responding to Recombinant IFN-γ

	Type of Response	Pretherapy Median (Range)	Posttherapy* Median (Range)
Hemoglobin (g/dL)	PR†	12.2 (10.8-13.9)	13.7 (11.2-16.9)
	CR‡	13.0 (11.4-16.1)	12.8 (10.8-14.0)
WBC ($\times 10^3/\mu L$)	PR	80 (25-99)	14.5 (10-19)
	CR	54 (14-98)	3.5 (2.0-7.3)
Platelets ($\times 10^3/\mu L$)	PR	660 (191-848)	300 (231-580)
	CR	495 (322-857)	290 (75-350)
Bone Marrow Blasts (%)	PR	6.2 (0.4-12.7)	5.0 (3.2-9.0)
	CR	10 (2.5-14.6)	3.2 (1.2-5.0)

Abbreviations: PR, partial remission; CR, complete remission.

*At time of maximal response.

†Number of patients with PR, 4.

‡Number of patients with CR, 6.

complete responders have shown evidence of cytogenetic improvement, with emergence of normal diploid metaphases in the bone marrow (Table 2). At the time of therapy initiation, the complete responders had favorable disease characteristics in that severe thrombocytosis or splenomegaly was not present. The median time from diagnosis of the complete responders was 10 months (range, 1 to 54 months). Prior therapy included the following agents: hydroxyurea (three patients), busulphan (three patients), Adriamycin-containing regimen (one patient); and no prior therapy (two patients). The median duration of prior therapy was 2 months (range, 0 to 11 months).

Control of thrombocytosis was achieved in all four patients, with baseline platelet counts $>1{,}000 \times 10^3/\mu L$. In these patients, the median (range) pretherapy platelet count was $1{,}200 \times 10^3/\mu L$ (range, 1,112 to $1{,}458 \times 10^3/\mu L$); the median (range) posttherapy nadir count was $450 \times 10^3/\mu L$ (range, 220 to $650 \times 10^3/\mu L$). However, the WBC counts of these patients remained elevated. Conversely, hydroxyurea had previously been able to control their WBC counts but not their thrombocytosis.

Five of 26 evaluable patients had prior treatment with IFN-α: three patients with rIFN-α (IFN-α-2a, Hoffmann-LaRoche, Inc, Nutley, NJ) and two patients with partially purified IFN-α (Cantell Finnish Red Cross, Helsinki). Of these five patients, one has achieved a complete hematologic remission with rIFN-γ therapy; two, a partial hematologic remission (Table 3). Five patients who failed rIFN-γ therapy were subsequently treated with rIFN-α. Two of these patients have had a complete hematologic response; one, a partial hematologic response.

Side effects. Most patients tolerated rIFN-γ treatment with minimal change in performance status. Although fever, night sweats, and flulike symptoms occurred in the majority of patients (Table 4), partial tachyphylaxis generally developed within 1 to 2 weeks. About one third of patients experienced mild myalgias. One patient who had a history of confusion after rIFN-α therapy became confused on day 10 of rIFN-γ treatment. Computerized tomographic scan of the brain was normal, and an EEG showed only diffuse brain wave slowing. Recovery occurred within 2 weeks after discontinuation of rIFN-γ injections. Elevation in liver enzymes to two to three times baseline levels occurred in approximately one third of patients. Consistent with the results of our previous studies, rIFN-γ had a marked effect on lipid metabolism, with hypertriglyceridemia occurring in virtually all patients.[26] These changes were reversible within 2 weeks after stopping IFN administration. A >30% reduction in platelet counts was noted in all patients. Even so, neither neutropenia (granulocyte count $\leq 1 \times 10^3/\mu L$) nor thrombocytopenia (platelet count $\leq 50 \times 10^3/\mu L$) was observed.

IFN antibody. Baseline and follow-up data from the radioimmunoprecipitation assay were available on 18 patients at a total of 56 time points. No antibody to rIFN-γ was detected.

Table 2. Changes in the Percentage of Ph-Positive Bone Marrow Cells Among CML Patients Who Achieved Complete Hematologic Remission

Patient No.	Ph-Positive Metaphases (%)				
	Pretreatment	3 mo	6 mo	9 mo	12 mo
1	100	80	—	50	55
2	100	—	80		
3	100	90	90	95	75
4	100	100	70		
5	100	100	100	95	
6	100	100			

Table 3. Response of CML Patients Who Are Resistant to One IFN to an Alternate IFN

Sequence	No. of Patients			
	Total	Response to Interferon		
		CR	PR	NR
α → γ	5	1*	2*	2*
γ → α	5	2†	1†	2†

Abbreviations: CR, complete hematologic remission; NR, no response; PR, partial hematologic remission.

*Response to rIFN-γ.

†Response to rIFN-α.

DISCUSSION

Single-agent chemotherapy can control the proliferative thrust of benign-phase CML but does not suppress the Ph chromosome or alter the inevitable progression towards blast transformation.[27] Aggressive combination chemotherapy results in a reproducible but transient decrease in the Ph chromosome level.[28-30] The majority of patients are ineligible for the only potentially curative therapy: supralethal chemotherapy and allogeneic bone marrow transplantation.[31,32] Therefore, exploration of other possible therapeutic avenues is important.

Previously, we have demonstrated that rIFN-α can induce hematologic remissions and cytogenetic improvement in newly diagnosed benign-phase CML patients.[12] Although IFN-γ does not bear significant homology to IFN-α, the in vitro suppressive effects of these two agents on myeloid colonies are similar.[13,14,33] Furthermore, McGlave et al[33] have recently demonstrated that in parallel studies of CML progenitor cells cultured with and without IFN-γ the presence of interferon resulted in a substantial increase in the

Table 4. Side Effects Experienced by 30 CML Patients Treated With rINF-γ

Side Effect	No. Patients (%)
Fever (>1.3°C)	30 (100) [4]
Night sweats	25 (83)
Chills	20 (66)
Myalgias	20 (66)
Headaches	14 (46)
Confusion	1 (3) [1]
Hypertriglyceridemia*	22 (73)
Hepatic toxicity†	10 (33)

Numbers in brackets indicate the numbers of patients in whom side effects were severe.

*Triglyceride levels increased to ≥1.5-fold baseline levels.

†SGOT levels increased to twofold baseline levels.

emergence of Ph-negative metaphases. These data prompted us to investigate the clinical effects of rIFN-γ in CML patients.

The present study indicates that approximately 25% of benign-phase, Ph-positive CML patients will attain a complete hematologic remission when receiving rIFN-γ therapy. Our experience with rIFN-α indicates that approximately 40% of complete responders will show cytogenetic improvement.[12] However, reemergence of diploid cells in the bone marrow requires at least 3 to 12 months of continuous treatment. Furthermore, the reduction in Ph-positive metaphases is ongoing, with maximal responses often observed in the second year of therapy.[34] Therefore, it was of interest that all five complete responders treated with rIFN-γ for ≥ 6 months demonstrated the reappearance of normal diploid metaphases in the bone marrow. Because at present the median duration of treatment is only 7.5 months, a longer follow-up of these patients will be essential to ascertain whether ongoing improvement will occur, as in the case of IFN-α.

Thrombocytosis occurs frequently among CML patients.[35] The morbid nature of severe thrombocytosis can be manifested clinically as thromboembolic and hemorrhagic phenomena.[35,36] Although control of the WBC count can be achieved with chemotherapeutic agents such as hydroxyurea, the concomitant control of platelet counts often becomes increasingly difficult. One to 5 weeks of rIFN-γ therapy resulted in normalization of platelet counts in all four patients with baseline counts $\geq 1{,}000 \times 10^3/\mu L$. However, significant cytoreduction of peripheral blood leukocytes was not achieved in these patients. We have previously described a similar phenomenon in CML patients treated with rIFN-α.[37] An increase in the autonomous proliferative capacity of platelet precursors has been suggested as an indicator of disease progression in CML.[38] Therefore, the discrepancy in control of platelets and WBC counts in these patients may reflect the association of more advanced disease with emergence of a resistant malignant clone. Interestingly, these patients' leukocyte and platelet counts responded differently to chemotherapy and IFN; hydroxyurea controlled the WBC count but not the platelet count. These data suggest that a combination of IFN and hydroxyurea may be beneficial in CML patients with severe thrombocytosis.

Side effects of rIFN-γ usually consisted of fever, chills, night sweats, and myalgias, with tachyphylaxis often occurring after the first week of therapy. These symptoms were qualitatively similar to those experienced by patients taking IFN-α. However, the severe neuromuscular pain occasionally described in IFN-α–treated CML patients[37] was not observed after rIFN-γ therapy. Most patients were able to tolerate 0.25 to 0.5 mg/m^2/d of IM rIFN-γ with minimal disruption of their usual daily activities.

Neutralizing antibodies to rIFN-α occur in approximately 10% of patients treated with this agent and may be associated with abrogation of some of its biologic activities.[39] In contrast, antibody to rIFN-γ was not detected in any patient in this study. Further, anti–rIFN-γ antibody has not developed in any of the approximately 300 patients treated with this agent and tested for antibody (Genentech, Inc, data on file).

In conclusion, the present study indicates that rIFN-γ has clinical antitumor activity in CML. Furthermore, it is possible for patients who are resistant to IFN-α to respond to IFN-γ, and vice versa. On the basis of these results and the in vitro synergistic antiproliferative effects of the two IFNs,[13] we have initiated studies of combination IFN therapy in CML.

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Clinical and Biologic Hallmarks of the Philadelphia Chromosome in Childhood Acute Lymphoblastic Leukemia

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Of 366 children with acute lymphoblastic leukemia (ALL) in whose bone marrow cells complete G-banding of chromosomes was successful, translocations were present at diagnosis in 141 (38.5%). The Philadelphia (Ph) chromosome was identified in 18 of these cases (4.9%). Features closely associated with the presence of the Ph chromosome were older age (median, 7.9 years), high leukocyte count (median, 47.3×10^9/L), French-American-British L1 blast cell morphology, high incidence of CNS leukemia at diagnosis, and the common ALL immunophenotype. All patients were treated according to modern chemotherapeutic regimens for ALL used at our institution. Complete remissions were successfully induced in only 13 (72%) of the 18 patients with Ph+ ALL, and only six remain free of leukemia for periods of 7+, 9+, 10+, 13+, 49+, and 70+ months. Our findings confirm the association of the Ph chromosome with classic high-risk features of ALL in children and suggest that this abnormality confers a very poor prognosis that has not yet been improved by modifications in established therapeutic regimens.

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ANALYSES OF AN ARRAY of clinical and biologic features have disclosed an unanticipated heterogeneity in acute lymphoblastic leukemia (ALL). Among the abnormalities discerned by cytogenetic analyses, structural rearrangements involving reciprocal exchanges of DNA are particularly interesting, not only because of their relatively high incidence rate but also because of their close association with clinical and immunologic characteristics. More recently, we and others have shown that translocations exert an independent adverse prognostic influence.[1,2]

The Philadelphia (Ph) chromosome, first described by Nowell and Hungerford[3] and subsequently characterized by means of chromosome-banding techniques as a balanced translocation, t(9;22)(q34;q11), has particularly novel features.[4] Although originally considered to be a cytogenetic marker specific for chronic myelogenous leukemia (CML), the Ph chromosome has been reported to be associated with other hematopoietic disorders including ALL in adults and children.[5] Much attention has been paid to this abnormality and focuses primarily on its relationship to the process of malignant transformation. Recent studies have indicated that the human homologue of the Abelson leukemia virus oncogene (*c-abl*) is located at band 9q34 and is translocated to band 22q11 in CML.[6] In addition, it has been shown that the *c-abl* gene product from CML cells is an aberrant mRNA that contains sequences derived from both chromosome no. 22 and chromosome no. 9.[7,8] Furthermore, this hybrid gene encodes a 210,000-dalton protein (p210) that has tyrosine kinase activity, thereby leading to speculation that it might function as a growth factor receptor.[9]

The unfavorable prognostic influence of the Ph chromosome in childhood ALL has been suggested previously.[10-13] We describe here the clinical and biologic characteristics and outcome of 18 children and adolescents with Ph+ ALL. Our results confirm the association of Ph+ ALL with established clinical and biologic risk factors and the poor treatment response of these patients in our experience.

MATERIALS AND METHODS

Patients and therapy. Six hundred ninety-eight consecutive patients with newly diagnosed ALL were admitted to St Jude Children's Research Hospital from November 7, 1978, to March 1, 1986. From 1983 to the present, chromosomes have been prepared by a direct bone marrow technique for ALL with a >90% recognition of clonal abnormalities and >95% complete and successful studies.[14] From 1978 to 1982, earlier versions of the technique were in use, with gradually increasing percentages of accurate identification of abnormal clones. During this earlier period, either no chromosome studies were done, or inadequate bone marrow specimens were available for approximately 20% of patients. Overall, successful chromosomal studies were performed for 80% of patients, and G-banding of all chromosomes was complete for 366 patients. This analysis was restricted to only those 366 patients with complete G-banding of chromosomes to avoid possible underrepresentation of clonal abnormalities. Time-to-failure analysis showed no significant differences between those patients whose chromosomes were fully banded and those whose were not ($P = .24$, data not shown). The patients were treated in one of three consecutive institutional therapeutic trials for ALL: ten in Total Therapy Study IX,[15] 200 in study X,[16] and 156 in study XI.[17] Each of these therapies consisted of modern intensive multiple-drug induction regimens, CNS prophylaxis, and 2½ years of multiple-drug continuation treatment. Because of the comparatively short follow-up time for patients in study XI, only the 210 patients enrolled in the two earlier studies whose follow-up period was adequate (median follow-up, 3.6 years) were included in the time-to-failure analysis.

At the time of this analysis, July 1986, 98 of the 210 patients have relapsed. The distribution of surviving patients included in the time-to-failure (interval from remission-induction to relapse or death) analysis was 0 to 2 years, 6 patients; 2 to 3 years, 43 patients; 3 to 4 years, 43 patients; more than 4 years, 20 patients.

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Excluded from the analysis were nine patients with B cell ALL, as defined by the t(8;14); they received a separate, very intense chemotherapy.[18]

Cytogenetic analysis. Chromosomes were prepared by a direct bone marrow technique developed here for ALL.[14] Briefly, bone marrow cells were collected in RPMI 1640 (Grand Island Biological Co, Grand Island, NY) medium supplemented with 30% fetal calf serum (FCS; Flow Laboratories, McLean, VA). The cells were exposed to colcemid (Grand Island Biological Co) (0.06 $\mu g/\mu L$) for 25 minutes before exposure to hypotonic KCl (0.075 mol/L) for 32 minutes. Cells were then fixed in 3:1 (vol/vol) methanol–acetic acid for 25 minutes, and slides were prepared by an edging-flame technique. G-banding was done on aged slides by treatment with trypsin and staining with Wright's stain (Sigma Chemical Co, St. Louis).

For each case, ten to 35 metaphases (mean, 20) were studied by direct microscopy to determine the modal number and identify the malignant cell line(s). Karyotypes were prepared from photographic prints. Chromosomal abnormalities were classified according to international conventions.[19] The definition of an abnormal stem line was that proposed by the Second International Workshop on Chromosomes in Leukemia.[20]

Hematologic and immunophenotypic studies. The diagnosis of ALL was based on the French-American-British (FAB) criteria for morphological classification[21] and negative myeloid-associated cytochemical reactions.[22] Immunophenotypes, determined by published methods,[23] confirmed the diagnosis of ALL. CNS leukemia at diagnosis was determined by the presence of leukemic blast cells in a cytocentrifuged specimen of CSF.

Statistical analysis. The chi-square test for contingency tables was used to compare differences in the distribution of clinical features and relapse rates for patients with or without the Ph chromosome. The analysis-of-variance techniques was used to compare the mean values of presenting clinical features that were continuous variables. A logistic regression model was used to identify factors that were significantly associated with failure to induce remission. Time-to-failure curves were constructed by the Kaplan-Meier procedure, and the intervals to failure were compared by the Cox-Mantel test. Time to failure was defined as the interval between remission induction and relapse or death from any cause. Patients in whom remission could not be induced were assigned a failure time of 0.

RESULTS

Of 366 children with ALL and complete G-banded blast cell karyotypes, 18 (4.9%) were found to have the Ph chromosome. Their clinical characteristics at diagnosis are shown in Table 1. The median age was 7.9 years (range, 2.1 to 18.4 years), and there were ten males and eight females. Sixteen patients were white, one was black, and one was Oriental. The median leukocyte count was 47.3 × 10^9/L (range, 2.0 to 545 × 10^9/L), the median hemoglobin was 8.9 g/dL (range, 3 to 13.7 g/dL), and the median platelet count was 46.5 × 10^9/L (range, 3.0 to 384.0 × 10^9/L). Eight patients had extramedullary disease at diagnosis: seven in the CNS and one in the mediastinum.

Two patients' cells had FAB L2 morphology, and 16 had FAB L1. Immunophenotyping studies indicated that 11 patients had common ALL. Blast cells from two patients formed heat-stable E rosettes characteristic of T cell ALL (patients no. 4 and 8), whereas those from three others contained cytoplasmic immunoglobulin indicative of pre-B ALL (patients no. 13, 15, and 18). For the remaining two patients, immunophenotyping studies were not available.

Comparison of presenting clinical and biologic features of patients with Ph+ ALL and Ph− ALL disclosed that patients with Ph+ ALL were more likely to be older ($P = .05$) and to have higher WBC counts ($P = .02$) than patients with Ph− ALL. In addition, CNS leukemia was present at diagnosis in seven of 18 children with Ph+ ALL as compared with 8.8% in the group without the Ph chromosome ($P < .01$). There was no difference between the two groups with regard to sex, spleen or liver size, hemoglobin content, platelet count, immunophenotype and FAB morphology (data not shown).

Cytogenetic studies (Table 2) disclosed the classic t(9;22)(q34;q11) in eight cases, a del(22)(q11) in seven

Table 1. Clinical Characteristics of Patients With Ph+ ALL

Patients	Age (yr)	Race	Sex	WBC (× 10^9/L)	Hemoglobin (g/dL)	Platelets (× 10^9/L)	Extramedullary Disease	Immuno phenotype/FAB Type	Remission Induced
1	9.5	W	F	4.5	7 8	260		CALLA+, L1	Yes
2	3.8	W	M	19.0	4.4	48		CALLA+, L1	Yes
3	18.4	O	F	335.0	4.2	25		CALLA+, L2	No
4	16 1	W	M	120.0	11.0	40	MM	T cell, L1	Yes
5	12.8	B	M	2.0	3.8	20		CALLA+, L1	No
6	5.1	W	F	6.7	11.8	384		NA, L1	Yes
7	3.2	W	F	28.5	8.8	3		CALLA+, L1	Yes
8	8.0	W	M	66.4	13.7	119	CNS	T cell, L1	Yes
9	6.8	W	F	316.0	3.0	40	CNS	NA, L1	No
10	3.7	W	M	66.1	11.1	30	CNS	CALLA+, L1	Yes
11	7.8	W	M	545.0	6.6	100	CNS	CALLA+, L1	No
12	6.7	W	F	345.0	5.7	45	CNS	CALLA+, L2	Yes
13	2.1	W	M	332.0	10.0	48		Pre-B, L1	Yes
14	17.4	W	F	20.3	9.8	15		CALLA+, L1	Yes
15	12.4	W	F	194.1	5.5	21	CNS	Pre-B, L1	No
16	10.1	W	M	8.3	9.9	359		CALLA+, L1	Yes
17	3.9	W	M	17.1	9.0	249	CNS	CALLA+, L1	Yes
18	15.0	W	M	3.0	9.0	226		Pre-B, L1	Yes

Abbreviations: MM, mediastinal mass; NA, not available, CALLA, common ALL antigen.

Table 2. Karyotypes of Patients With Ph+ ALL

Patient	Karyotype
1	46,XX,del(22)(q11)
	45,X,−X,−9,+mar,del(22)(q11)*
2	46,XY,+3,−C,del(22)(q11)
	46,XY,del(22)(q11)*
3	46,XX,t(9;22)(q34,q11)
4	46,X,del(Y)(q12),del(22)(q11)
	52,X,−Y,+6,+9,+del(10)(q24),+11,+13,+20,+mar, del(12)(q23),del(22)(q11)*
5	47,XY,+del(22)(q11)/49,XY,+4,+16,+del(22)(q11)
6	54,XX,+dup(7)(q22q36),+del(9)(q31),+14,+15,+21, +21,+del(22)(q12),+mar
7	46,XX,del(22)(q11)
	48,XX,+C(?6),+7,t(7;20)(q22;q13),?t(8;9,22) (p21;q34;q11)*
8	46,XY,idic del(22)(q11)
9	46,XX,del(22)(q11)
	46,XX,del(22)(q11)*
10	46,XY,−19,+der(19),t(1;19)(q23;p13.3),t(9;22) (q34,q11)
	46,XY,t(9;22)(q34;q11)*
11	46,XY,t(9;22)(q34;q11)
12	48,XX,+8,+10,t(9;22)(q34;q11)/ 48,XX,+8,+10,del(9)(p22),del(8)(p22),t(9;22) (q34;q11)
13	54,XY,+X,+2,+5,+6,+14,+18,+21,+del(22)(q11), t(9;22)(q34;q11)
14	53,XX,+X,+2,+6,+14,+18,+21,+del(22)(q11), t(9;22)(q34;q11)
15	46,XX,t(9;22)(q34;q11),del(10)(p14),inv(12)?(p11p13), del(17)(p13)
16	46,XY,t(9;22)(q34;q11),del[der(9),t(9;22)](p13)/ 46,XY,t(9,22)(q34;q11),−9,+der(9),t(9,?)(p24;?), del[der(9),t(9;22)](p13)/46,XY,t(9;22)(q34;q11),−19, +der(19),t(19;?)(p13;?)
17	46,XY,t(6;9;15;22)?(q21;q21;q22;q11)
18	94,XY,+2,+del(2)(p21),+3,−?7,2xder(12),t(12;?) (p13;?),−?13,−?14,−?15,+19,+21,+mar, +2xdel(22)(q11),2xdel(22)(q11)

*Karyotypes at relapse.

cases, a del(22)(q12) in one case, the rare variant idic del(22)(q11)(isodicentric Ph) in one case, and a complex translocation involving chromosomes no. 6, 9, 15, and 22 in another case. The modal number of chromosomes was 46 in 12 patients, whereas six were hyperdiploid (47, 48, 53, 54, 54, and 94). Serial cytogenetic studies were performed on bone marrow cells from five of the 13 patients who were in remission for 3 to 10 months. In four cases, normal karyotypes were identified without any evidence of the Ph chromosome. When tested after 2 months of clinical remission, the remaining patient retained a clone that was negative for the Ph chromosome but had a deletion of chromosome Y that had been noted at diagnosis. Three of these five patients subsequently relapsed with reappearance of the Ph chromosome (patients no. 1, 2, and 4). In addition, two other patients studied again only at relapse had the Ph chromosome.

Complete remission was induced in 13 (72%) of the 18 patients with Ph+ ALL. Of the five patients in whom remission was not induced, two had drug-resistant disease and persistent leukemia, and three died of infection or metabolic complications. Of the 13 patients in whom remission was attained, seven have relapsed: three in the marrow, three in the CNS, and one combined bone marrow and CNS relapse. Six patients remain in continuous complete remission for periods of 7+, 9+, 10+, 13+, 49+, and 70+ months. Of these six patients, the two who are considered long-term survivors (49+ and 70+ months) had variant forms of the Ph chromosome (patients no. 6 and 8, Table 2).

The Kaplan-Meier plots of time to failure for the 210 patients who had adequate follow-up times are shown in Fig 1. Clearly, there is a significantly increased risk of treatment failure for patients with the Ph chromosome and for those with other translocations compared with ALL patients without chromosomal translocations. The group with translocations included 46 patients with random and 24 patients with nonrandom translocations (three patients had t(4;11), 17 had t(1;19), and four had t(11;14)). Because there were so few patients in each category, it was impossible to compare treatment response for each translocation. Although not included in the analysis, the treatment response of patients with B cell ALL and the 8;14 translocation has been similar to those with Ph+ ALL, and there was no significant difference in time to failure between the two subgroups ($P = .36$, data not shown).

DISCUSSION

Our chromosomal analyses confirmed the relative infrequency of the Ph chromosome in children with ALL that largely accounts for the difficulty in establishing the clinical and biologic characteristics of Ph+ childhood ALL. The incidence rate of this abnormality was 4.9% in this group of children, similar to that found in our previously published study of 161 patients.[2] The Third International Workshop on Chromosomes in Leukemia[11] found nine (5.7%) patients with the Ph chromosome among 157 childhood ALL patients with completely banded chromosome studies. In a smaller series,

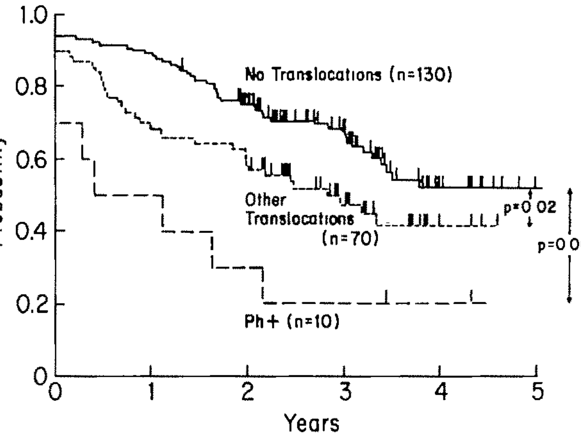

Fig 1. Comparison of time-to-failure intervals according to presence or absence of translocations. Significantly worse treatment results were evident for patients with the Ph chromosome and other translocations excluding t(8;14) ($P = .0025$ and $P = .020$, respectively, by the Cox-Mantel test).

Priest et al[10] determined that the Ph chromosome was present in four (9.3%) of 43 patients. Secker-Walker et al[24] and the Pediatric Oncology Group[12] reported lower incidences of 2% (four of 120 patients) and 1.4%, respectively. This variation most likely reflects differences in patient selection criteria or in rates of successful chromosome studies. In the study by Priest et al,[10] all children with Ph+ ALL were ≥10 years old, possibly reflecting a larger proportion of older children in that group, whereas Secker-Walker et al studied only those patients whose blast cells could not be classified by morphological, cytochemical, or immunologic methods or patients in whom remission was not induced after 2 months of therapy. We recently reported successful banding of chromosomes from more than 90% of patients with ALL.[25] Thus, this analysis of consecutive patients should allow a more accurate assessment of the incidence of childhood Ph+ ALL.

The association of Ph+ ALL with clinical and biologic features known to be indicative of poor prognosis has led many investigators to suggest that the poor outcome of this group might be explained, at least in part, by associated high-risk features such as a high leukocyte count.[12] However, others have indicated that the Ph chromosome exerts an adverse influence on prognosis independent of any other high-risk feature.[13] In our series, 14 patients had at least one classic high-risk feature. As a group, Ph+ ALL patients were more likely to be older and to have higher WBC counts and an increased incidence of CNS leukemia compared with patients with Ph− ALL. Particularly striking is the significantly increased incidence of initial CNS leukemia in this group (seven of the 18 children). We recently reported a group of 161 patients in whom chromosomal translocations exerted an adverse prognostic influence independent of traditional risk features. The results of this study further confirm our earlier observations and suggest that the response to therapy may vary among the various translocation categories. However, because there were relatively few patients in each category, we were unable to determine more precisely by multivariate analysis the independent prognostic importance of individual translocations or to correct for the possibly confounding effect of individual treatment variables over successive therapeutic protocols spanning 8 years.

Of interest are the two patients who are long-term survivors who had variant forms of the Ph chromosome (patients no. 6 and 8, Table 2). Although the significance of these cytogenetic variations is unknown, they raise potentially important considerations regarding the microheterogeneity of DNA rearrangements observed in leukemic cells. It has been shown recently that nonrandom translocations involving band q11 of chromosome no. 22 are numerous and associated with a variety of malignancies as well as constitutional disorders.[26] In addition, these translocation breakpoints, although morphologically indistinguishable, can be discerned with respect to the chromosome locus by use of in situ hybridization.[27] Moreover, this molecular heterogeneity may also have clinical relevance. It has been shown that specific chromosomal rearrangements in leukemic cells are related to the type of disease,[28] disease progression[29] and prognosis. Whether variant forms of the Ph chromosome are associated with malignant cells having less proliferative capacity or lower drug resistance remains to be determined.

Clinically, the distinction between Ph+ ALL and Ph+ CML in lymphoblastic crisis can be a difficult one. Most patients presenting with ALL whose blast cells contained the Ph chromosome were originally thought to have CML in lymphoblastic crisis. However, in the series of patients we report, complete remission was induced in 13 patients whose subsequent bone marrow morphology was apparently normal, unlike most patients with CML. Also, the Ph chromosome was not present in metaphases from five patients in clinical remission. Because patients with CML in blast crisis usually retain the Ph chromosome abnormality while in clinical remission and show characteristic abnormalities of blast crisis,[5] it seems unlikely that a diagnosis of CML in lymphoblastic crisis would be accurate for these five patients. In addition, the clinical features of patients with Ph+ ALL at diagnosis are much more similar to de novo childhood ALL than to CML in lymphoblastic crisis. None of the patients we describe had a previous history of hematopoietic abnormalities.

The findings of recent investigations suggest that there are also molecular disparities between CML and Ph+ ALL.[30-32] In a majority of patients with CML, the breakpoints of chromosome no. 22 lie within a 5.8-kilobase (kb) span referred to as the breakpoint cluster region (*bcr*).[33] Additionally, CML cells express an aberrant 8.0-kb *c-abl* transcript.[8] In contrast, the breakpoints of chromosome 22 in cells from two patients with Ph+ ALL studied by Rodenhuis et al[30] were not in or near the *bcr*, and the breakpoints in Ph+ ALL cells from two patients reported by Cannizzaro et al were located proximal (5′) to the *bcr*.[34] More recently, a novel 6.5-kb *c-abl* transcript that encodes a unique 185,000-dalton protein (p180) with tyrosine kinase activity has been found in leukemia cells from patients with Ph+ ALL.[32] This protein is distinct from the p210 protein typically associated with the chronic phase and blast crisis of CML. This evidence suggests that Ph+ ALL might involve pathogenetic mechanism(s) distinct from that of Ph+ CML. If the findings of this latter study are verified in a larger patient population, it is possible that Ph+ ALL could be definitively differentiated from the blast crisis of CML by rapid screening for p185 or p210 protein expression using immunofluorescence technology, cell sorting, and Western blots.

In our experience, the presence of the Ph chromosome confers a very poor prognosis that apparently has not been improved by modifications in intensive multidrug chemotherapeutic regimens. The curative role of bone marrow transplantation for some patients with Ph+ CML has been established.[35] Less clear is the efficacy of this treatment modality for Ph+ ALL. Because of their poor response to conventional therapy, alternative treatment methods such as bone marrow transplantation should be investigated for this group of patients.

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Characterization of Interleukin-2 Receptors Expressed on Acute Leukemic B Cells

By Martine Bentaboulet, Michèle Allouche, Andreas Tsapis, Claude Jasmin, and Vassilis Georgoulias

More than 90% of both unfractionated and blast-enriched T cell–depleted mononuclear cells (MNC) from a patient with a morphologically and phenotypically unclassified acute leukemia expressed interleukin 2 receptors (IL2-R), whereas 50% unfractionated MNC displayed monocyte/myeloid- (My7, My9, and OKM1) and B cell–restricted (B4) antigens. Two-color fluorescence studies showed that 80% of the My9+ cells expressed the B4 antigen and 63% of the B4+ cells were IL2-R+.

Cell incubation with phorbol myristate acetate (PMA) increased the expression of B4 antigen and significantly decreased the proportion of My9+ and IL2-R–bearing cells. Southern analysis of DNA from leukemic cells revealed monoclonal rearranged heavy- and k light-chain immunoglobulin genes. Immunoprecipitation of leukemic cell membrane proteins with a monoclonal antibody recognizing the IL2-R (anti-Tac) revealed a protein with the same molecular weight (55 kilodaltons) as that of the IL2-R present on PHA-stimulated normal T cells. Fresh leukemic cells did not express high-affinity IL2-R and did not proliferate in liquid culture or in a colony assay in the presence of recombinant IL2 (rIL2). PMA-treated blast cells, however, generated B cell colonies in the presence of rIL2, thus suggesting that PMA could induce functional IL2-R on immature leukemic B cells.

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NORMAL ACTIVATED T LYMPHOCYTES produce interleukin 2 (IL2), which provides a necessary signal for the proliferation of T cells.[1-3] IL2 binds to a high-affinity receptor on the surface of responsive cells.[3,4] The murine monoclonal antibody (MoAb) anti-Tac, which recognizes IL2 receptors (IL2-R),[5,6] can block all of the T cell responses attributed to interaction of IL2 with its receptor on these cells.[7] However, quantitative binding assays with radiolabeled IL2 and anti-Tac showed that normal activated T cells had ten to 20 times more binding sites for the anti-Tac than high-affinity sites for IL2.[4] These additional binding sites represent low-affinity IL2-R,[4] which are also expressed on human leukemic T cell lines.[8,9]

Recent studies have demonstrated that IL2 is also involved in the proliferation of normal activated murine[10] and human[11-14] B cells that also express IL2-R. Anti-Tac MoAb precipitates a protein of the same molecular weight (mol wt) (55 to 56 kilodaltons [kD]) from both normal activated B and T cells.[14-16] IL2-R are expressed on activated normal B cells with a lower density and affinity than on activated normal T cells and with a different kinetics of appearance.[15] Moreover, Korsmeyer et al recently described both IL2-R expression and immunoglobulin gene rearrangement in human hairy cell leukemia cells.[17]

In this report we describe a case of morphologically unclassified acute leukemia, which by molecular biology and phenotypic studies appears to be of B cell origin. The leukemic cells expressed IL2-R of the same mol wt as those expressed on normal activated T cells.

PATIENT AND METHODS

Case report. A 49-year-old man was admitted in September 1984 because of abdominal pain. On physical examination, he presented with generalized lymphadenopathy, hepatomegaly, and splenomegaly A peripheral blood count revealed thrombocytopenia and hyperleukocytosis (WBC, 9.6×10^{10}/L), with 80% of the leukemic cells displaying a morphology compatible with that of hemocytoblasts. Cytochemical studies showed that the leukemic cells were myeloperoxydase-, Sudan black–, PAS-, acid phosphatase–, β-glucuronidase–, and esterase-negative but neutral esterase–positive (100% of the cells). Karyotypic analysis revealed no mitosis in unstimulated or pokeweed mitogen–stimulated cells, whereas phytohemagglutinin (PHA) stimulation demonstrated a normal karyotype. The patient was treated with combination chemotherapy including Aclacinomycine and cytosine arabinoside, entered profound aplasia, and died 1 month later of cerebral hemorrhage. For our study, peripheral blood from the patient was taken before treatment, with his informed consent.

Separation of mononuclear cells MNC were obtained by Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden) density centrifugation. In some experiments, MNC were further separated by sheep RBC (SRBC) rosette formation[18] and complement-mediated cytotoxicity with the OKT3 and OKT11 (Ortho Pharmaceutical Corp, Raritan, NJ) MoAbs as described.[19] This cell fraction, which contained less than 1% E+ and/or T3+ cells, will be referred to as E−T3−T11−.

MoAb Fresh MNC or E−T3−T11− cells were phenotyped by using the following MoAbs: (*a*) Myeloid/monocyte-associated: OKM1 (Ortho), My4, My7, and My9 (Coultronics, Margency, France); (*b*) T cell associated· the OKT series (Ortho) including the OKT3 (pan-T), OKT4 (helper/inducer), OKT8 (suppressor/cytotoxic), OKT6 (common thymocyte), and OKT11 (pan-T, recognizing the SRBC receptor); Leu 9 (Becton Dickinson, Buc, France) and WT1[20] (a gift from Dr W. Tax, Nijmegen, the Netherlands) (pan-T); (*c*) B cell–associated B1 and B4 (Coultronics) recognizing mature and immature/mature B cells respectively and the J5 (Coultronics), which recognizes the common acute lymphoblastic leukemia antigen; (*d*) multilineage: BM50 (anti-HLA class II, a gift from Dr D. Charron, Hôpital de la Pitié, Paris) and RFB1 (a gift from Dr G. Janossy, London) recognizing hematopoietic precursors, both immature and mature T but not B cells[21]; OKT9 (Ortho, antitransferrin receptor) and OKT10 (Ortho), which is expressed on immature T cells and other proliferating cells; and IOT14 (Immuno-

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tech, Luminy, France) and anti-Tac (kindly provided by Dr T.A. Waldmann, National Institutes of Health, Bethesda, MD), which recognize IL2-R. Cells were stained by indirect immunofluorescence using fluorescein isothiocyanate–coupled goat antimouse immunoglobulin (Nordic, Tilburg, The Netherlands) as a second reagent.

Surface immunoglobulin (sIg) was tested by direct immunofluorescence using FITC-coupled goat antihuman immunoglobulin (Cappel Laboratories, Cochranville, NJ).

Two-color fluorescence studies. Cryopreserved cells were studied by two-color fluorescence using MoAbs coupled to phycoerythrin (B4-RD1, Coultronics), fluorescein (Leu9-FITC), or biotin (IOT14-biotin); in the latter case, the second reagent was fluoresceinated or rhodaminated streptavidin (Jackson Laboratories, Bar Harbor, Maine). No background fluorescence was observed with any of the second reagents alone. For double labeling with My9 and B4, cells were first incubated with My9 followed by GAM-FITC; the third addition was phosphate-buffered saline containing 1% normal mouse serum to prevent nonspecific binding of the final addition, B4-RD1 (also a murine MoAb), to GAM-FITC Incubations were carried out for 15 minutes at 4°C and were separated by two washes. The samples were evaluated on a Leitz epifluorescence microscope equipped with a combination of blue (450 to 490 nm for FITC and phycoerythrin) and green (530 to 560 nm for rhodamine) excitation filters with stop filters at 515 and 580 nm, respectively. The percentage of positive cells obtained with a given MoAb was the same, regardless of the technique used (direct *v* indirect, single *v* double, green *v* red labeling).

Induction of differentiation by phorbol myristate acetate. A quantity of 10^6 MNC or E−T3−T11− cells/mL was incubated for 24 to 72 hours in growth medium (α–minimum essential medium [α-MEM], GIBCO, Grand Island, NY) containing 10% (vol/vol) heat-inactivated fetal calf serum (FCS) with increasing concentrations of phorbol myristate acetate (PMA) (10^{-1} to 10^3 ng/mL) (Sigma Chemical Co, St Louis). At the end of the incubation, cells were extensively washed, their viability was tested by Trypan blue dye, and the cells were stained with different MoAbs. Control cultures contained no PMA.

Southern analysis of immunoglobulin heavy- and light-chain gene rearrangement. DNA was extracted from frozen cells and digested with restriction enzymes (Boehringer Mannheim, Mannheim, West Germany) in appropriate buffer. Human placental DNA was used as a control. Ten micrograms of DNA digested with *Bam*HI were electrophoresed in 0.8% agarose gels for 36 hours. The DNA was transferred to Gene Screen Plus (NEN, Du Pont de Nemours, Paris) filters.[22] Filters were prehybridized in 10% dextran sulfate, 6× SSC, 5× Denhardt's, 0.5% sodium dodecyl sulfate (SDS), 5 mmol/L EDTA, and 100 μg/mL salmon sperm DNA at 65°C. Hybridization was performed for 16 hours in the same solution with the addition of a radiolabeled probe at a concentration of 5×10^6 cpm/mL. Radiolabeled probes were obtained by nick translation[23] in the presence of [α^{32}P]-dCTP (deoxy-cytioline triphosphate) (3,000 Ci/mmol; NEN) The specific activity of these probes was about 10^8 cpm/μg of probe The hybridized filters were washed four times in 2× SSC and 0.5% SDS at room temperature and then three in 0.1× SSC and 0.5% SDS at 65°C for 30 minutes. Filters were autoradiographed for 16 to 72 hours on Kodak XAR-5 films at −80°C with intensifying screens (Cronex Plus, Du Pont de Nemours).

Southern analysis of the T cell receptor β chain genes. DNA was digested by each of the three restriction enzymes *Eco*RI, *Bam*HI and *Hind*III, electrophoresed on agarose gel, transferred to nylon membranes, and hybridized as described earlier with the T cell receptor β chain (TcRβ) probe, which was radiolabeled by the random priming method.

Probes used. J_H was a 2.2-kilobase (kb) Sau 3A fragment of human genomic DNA containing the J_H region.[24] The C_k probe was a 2.5-kb *Eco*RI fragment of genomic DNA containing the constant region of the κ gene[25] These probes have been isolated from recombinant phages (kindly provided by Dr P. Leder, Boston).

The TcRβ probe was a 0.4-kb *Bgl*II fragment of genomic DNA containing the majority of the constant region of the TcRβ gene (a kind gift of Dr Strominger, Boston).

Proliferation assay. A quantity of 2×10^5 cells in growth medium (α-MEM, GIBCO) supplemented with 10% (vol/vol) FCS were seeded per well of a microtiter plate (Nunc, Roskilde, Denmark) in the presence or the absence of increasing amounts (0 1 to 10.0 French U/mL) of recombinant IL2 (rIL2) (a gift from Biogen, Geneva; 1 French unit ≃ 40 Biological Responses Modifiers Program units). After 48 hours incubation, cells were pulsed for an additional 18 hours with 1 μCi/well of ^{3}H-thymidine (^{3}HTdR; specific activity, 20 to 25 Ci/mmol; CEA, Saclay, France). Cells were havested with an automated cell harvester, and cell-associated radioactivity was counted by liquid scintillation.

Colony formation. A quantity of 5×10^5 cells/mL was seeded in 0.8% (vol/vol) methylcellulose (Fluka, Bachts, Switzerland) in growth medium supplemented with 15% (vol/vol) FCS and 2 mmol/L L-glutamine in the absence or the presence of various doses of rIL2. One-tenth milliliter of the methylcellulose cell–containing preparation was seeded per well in 96-well, flat-bottomed microtest plates and incubated at 37°C in 5% CO_2 in air for seven days Aggregates containing more than 50 cells were counted under an inverted microscope as colonies.

Binding of radiolabeled IL2. Biosynthetically labeled rIL2 was either ^{35}S-rIL2 (specific activity, 1,313 dpm/10^{-15}/mol/L; a gift of Dr Y. Jacques, Nantes, France) or ^{125}I-rIL2 (purchased from NEN, Du Pont de Nemours, France; specific activity, 20 to 50 μCi/μg). Binding of radiolabeled IL2 to MNC was performed according to Robb et al.[4] Briefly, 3×10^5 cells were incubated with serial dilutions of radiolabeled IL2 for one hour at 4°C, a control was performed in the presence of a 100-fold excess of cold rIL2 to determine the level of nonspecific binding The cells were then spun down through a mixture of dibutyl phtalate and paraffin oil (9/1 vol/vol, Sigma) at 9,000 g for two minutes, and radioactivity of the supernatant (unbound) and of the pellet (bound) was determined by liquid scintillation or in a γ-scintillation counter for experiments performed with ^{35}S-IL2 and ^{125}I-IL2, respectively

Immunoprecipitation. Cell membranes of intact cells were iodinated by the lactoperoxydase technique (1 mCi ^{125}I/10^7 cells).[26] Iodinated cells were lysed as described previously[27] The soluble material was recovered by centrifugation and incubated with 50 μL staphylococcal suspension (SAC I) for 30 minutes at 4°C IL2-R was immunoprecipitated from precleared supernatant with 2 μL anti-Tac–purified MoAb and 50 μL rabbit IgG antimouse IgG-coated SAC I. Washed, bound proteins were recovered by centrifugation and eluted with 50 μL of 6.25 mmol/L Tris-HCl, pH 6.8, 2% SDS, and 10% glycerol by boiling for three minutes. Samples were electrophoresed under reducing and nonreducing conditions on an SDS–7.5% polyacrylamide gel[28] and autoradiographed with Kodak XAR-5 film

RESULTS

Phenotype of leukemic cells. As shown in Table 1, unfractionated MNC were RFB1+, T10+, and 91% of the cells expressed IL2-R as defined by both IOT14 and anti-Tac MoAbs. Forty to 50% of the cells expressed myeloid/monocyte markers (My7, My9, and OKM1), and 57% were B4+. B1 antigen and sIg were present on only 12% and 15% of the cells, respectively. Forty-five percent of the cells

Table 1. Phenotype of the Patient's MNC

MoAb	Positive Cells (%)
Myeloid/monocyte associated	
OKM1	38
My4	12
My7	46
My9	49
T cell associated	
T3	8
T4	10
T8	8
T11	21
T6	4
WT1	23
Leu9	33
B cell associated	
B1	12
B4	57
J5	28
sIg*	15
Multilineage	
BM50 (HLA class II)	45
RFB1	98
T9	16
T10	82
IL2-R	91

*FITC-coupled goat antihuman immunoglobulin antiserum.

expressed HLA class II antigens (BM50). T cell specific markers (such as T11, WT1, and Leu 9) were expressed on less than 35% of the cells (Table 1). Two-color fluorescence studies revealed that 70% of the B4+ cells expressed the My9 antigen and 81% of the My9+ cells were also B4+. Moreover, 63% of the B4+ cells expressed the Tac molecule. When the T cell-depleted, blast-enriched, E−T11−T3− cell fraction that contained 2% WT1+ cells was examined, 95% of the cells were still found to express IL2-R.

Induction of cell differentiation with PMA. To define the cell origin of this leukemia, unfractionated MNC were induced to differentiate in vitro with PMA. A 24-hour incubation with PMA did not significantly modify the cell phenotype, except for an increase in B4 antigen expression (69% to 100% B4+ cells at 1 to 10^2 ng/mL of PMA). A 48-hour incubation with PMA resulted in 100% B4+ cells and in a decrease of IL2-R, sIg, My9, and T3 antigen expression, respectively (Fig 1A). Moreover, WT1+ cells represented less than 10% of the cells at all tested PMA concentrations (range, 4% to 10%). There was no PMA-induced cytotoxicity because more than 80% of the cells were viable in both control and PMA-induced cultures.

In addition, incubation of blast-enriched (E−T11−T3−) cells with 10 to 10^3 ng/mL PMA for 48 hours also resulted in the expression of the B4 antigen in 100% and IL2-R in 60% of the cells, respectively (Fig 1B).

Southern analysis of immunoglobulin heavy- and light-chain genes, and of TcRβ chain genes To investigate whether the leukemic cells were monoclonal committed B cells, analysis of human immunoglobulin genes was undertaken. The results obtained are represented in Fig 2A. Placental DNA showed a 17-kb J_H-positive band corresponding to the germline configuration of immunoglobulin heavy-chain genes, whereas the patient's cell lane exhibited two J_H-positive bands. One of them corresponded to the germline (17 kb), and one was rearranged (14 kb).

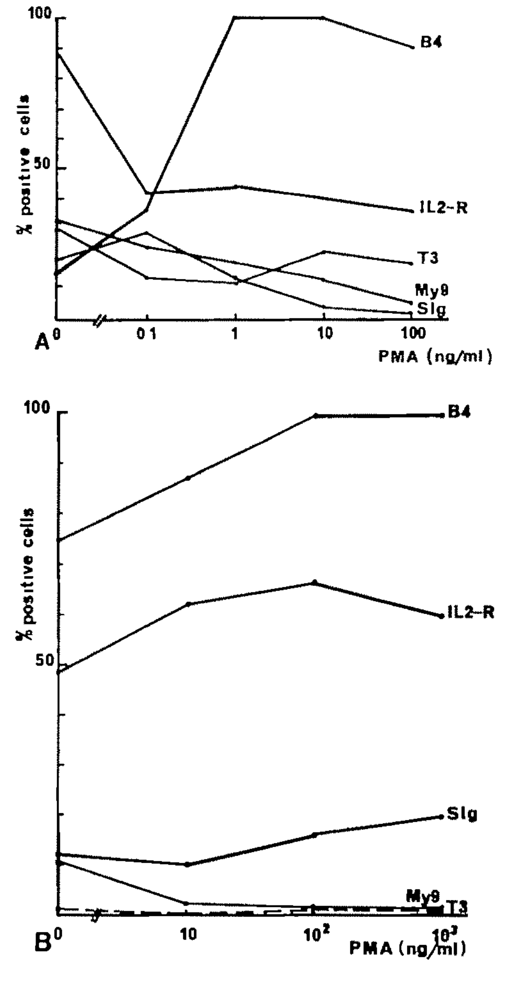

Fig 1. Induction of leukemic cell differentiation with PMA. The patient's unfractionated MNC (A) or T cell-depleted, blast-enriched (E−T3−T11−) cells (B) were incubated for 48 hours with various concentrations of PMA or control medium. The cells were then washed, and their phenotype was studied by using MoAbs recognizing B4, T3, IL2-R, My9, and one antiserum for human sIg.

*Bam*HI-digested DNA from both the patient's cells and placenta was analysed by using a constant κ region probe. The patient's DNA lane showed two bands, one germline (12 kb) and one rearranged (10 kb) (Fig 2B). The equal intensity of the two bands shows that the cells were monoclonally rearranged. The pattern of TcRβ-positive bands was that of germline genes, thus indicating an absence of rearrangement of the TcRβ genes (not shown).

Immunoprecipitation of the IL2-R expressed on leukemic cells. Cell membrane proteins, from both the patients' leukemic cells and removal activated T cells, were immunoprecipitated with purified anti-TAC MoAb. As shown in Fig 3, SDS–polyacrylamide gel electrophoresis (PAGE) analysis of the immunoprecipitates reveals a band with an apparent mol wt of 55 kD under reducing conditions for both leukemic

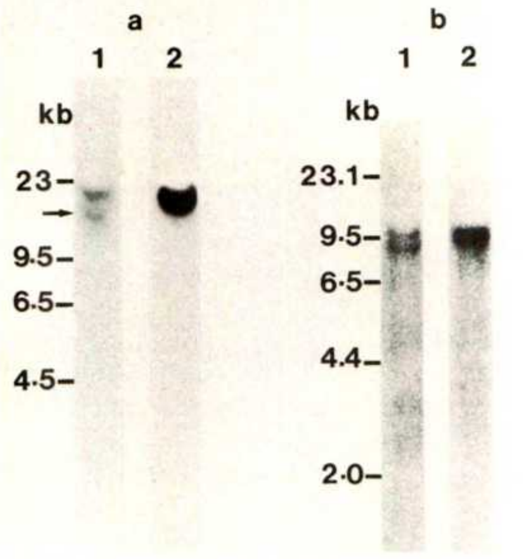

Fig 2. Ig gene configuration in acute leukemic cells. DNA isolated from the patient's cells was analyzed for Ig gene rearrangements by Southern hybridization. For heavy-chain gene analysis with the J_H probe (A) and for κ light-chain analysis with the C_κ probe (B), DNA was cleaved with the restriction endonuclease *Bam*HI. Arrows indicate the position of rearranged immunoglobulin bands: lane 1, patient's cellular DNA; lane 2, placental DNA.

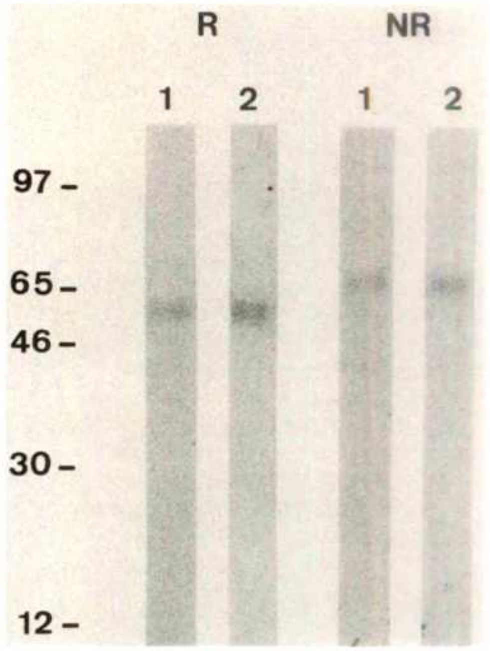

Fig 3. SDS-PAGE analysis of IL2-R expressed on leukemic cells under reducing (R) and nonreducing (NR) conditions after immunoprecipitation from lysates of ^{125}I-labeled cells with anti-Tac MoAb. Lane 1, patient's cells; lane 2, normal PHA-stimulated T cells. Size markers: 97, 65, 46, 30, and 12 kD.

and normal activated T cells. Under nonreducing conditions, the molecule also migrated as one band in both cases, but with an apparent mol wt of 65 kD, which suggests intrapolypeptide disulfide bond(s).

Responsiveness of IL2-R–bearing cells to rIL2. Because the patient's leukemic cells were of B cell origin and expressed IL2-R, it was of interest to determine whether they could proliferate in the presence of rIL2. rIL2 concentrations ranging from 0.02 to 2.0 U/mL could not induce either colony formation or ^{3}HTdR incorporation from unfractionated MNC (not shown). Moreover, the blast-enriched E−T3−T11− cell fraction did not respond to 0.1 to 10 French U/mL of rIL2 (Table 2). Conversely, PHA-lymphocyte-conditioned medium (a seven-day supernatant of PHA-M [1% vol/vol]-stimulated normal MNC) induced colony formation but no significant ^{3}HTdR incorporation by leukemic cells (Table 2). Phenotyping of pooled colony cells revealed 37% WT1+, 53% IOT14+, 26% B4+, and 81% My9+ cells.

To define whether the functional status of IL2-R expressed on leukemic cells could be modified, E−T3−T11− cells were incubated with either growth medium or 100 ng/mL PMA for 48 hours, washed, and seeded in methylcellulose in the presence and the absence of 0.5 U/mL of rIL2. As shown in Table 3, cell incubation in growth medium or PMA induced the generation of small colonies (up to 100 cells/colony) in the absence of added growth factors. The addition of rIL2 allowed the growth of large and round colonies that contained at least 10^3 cells. Moreover, rIL2 enhanced about twofold the plating efficiency of cells preincubated with PMA (Table 3). Colonies from cells preincubated with growth medium contained a relatively high proportion of IL2-R+, WT1+, and My9+ cells (53% to 80%, 37% to 42%, and 81% to 50%, respectively), whereas colonies from PMA-preincubated cells were of B cell origin (more than 50% B4+ cells and about 10% WT1+ and My9+ cells, Table 3). Spontaneous colonies from PMA-incubated cells contained cells bearing the IL2-R, but their proportion was decreased in rIL2-induced colonies (46% and 22% IL2-R+ cells, respectively; Table 3).

Binding of radiolabeled rIL2 to IL2-R–bearing leukemic cells. Binding experiments with radiolabeled rIL2, used at

Table 2. Colony Formation From Blast-Enriched Cell Fraction (E−T3−T11−)

Source of Growth Factor	Number of Colonies/ 5×10^4 Cells*	^{3}HTdR Incorporation (cpm/2×10^5 Cells)†
Control medium	0	1,365 ± 852
rIL2 (0.1-10 U/mL)‡	0	1,741 ± 356
PHA-LCM (10% vol/vol)	48 ± 8	2,758 ± 620

*A quantity of 5×10^5 cells/mL was seeded in 0.8% (vol/vol) methylcellulose in the presence of control medium, rIL2, or PHA-LCM as described in Materials and Methods. Aggregates containing >50 cells were counted as colonies after five to seven days of incubation at 37 °C, 5% CO_2.

†A quantity of 2×10^5 cells/0.2 mL growth medium was seeded in microtiter wells in the presence of control medium, rIL2, or PHA-LCM and incubated for 48 hours. They were pulsed with 1 μCi/well of ^{3}HTdR for an additional 18 hours, and cell-associated radioactivity was counted by liquid scintillation.

‡Serial twofold dilutions of rIL2.

Table 3. rIL2-Induced Colony Formation From PMA-Incubated, Blast-Enriched Leukemic Cells

Preincubation (2 d)	Added rIL2	Number of Colonies/ 5×10^4 Cells	Positive Colony Cells (%) WT1	IL2-R	B4	My9
Control medium	−	92 ± 11*	37	53	20	81
	+	130 ± 24†	42	80	26	50
PMA (10^2 ng/mL)	−	334 ± 26*	12	46	59	11
	+	794 ± 92†	9	22	68	3

A quantity of 10^6 E−T3−T11− cells/mL was incubated with growth medium (α-MEM) containing 10% FCS in the absence or presence of PMA (10^2 ng/mL) for 48 hours. Cells were extensively washed and seeded in methylcellulose with and without 0.5 U/mL rIL2. After five days, individual colonies were picked up, pooled, and dissociated, and washed cells were stained with various MoAbs by indirect immunofluorescence.

*Small colonies containing <100 cells.

†Large and round colonies containing up to 10^3 cells.

doses that gave a significant binding on 48-hour PHA-stimulated normal T cells (2 to 130 pmol/L of ^{35}S-rIL2) revealed no specific cell-associated ratioactivity on the patient's unfractionated MNC. In another experiment, cryopreserved MNC were thawed, incubated or not with 100 ng/mL PMA for 24 hours, and used in the binding assay; in both cases no specific binding was observed in the range of 50 to 1,000 pmol/L of ^{125}I-rIL2.

DISCUSSION

The results presented here indicate that leukemic cells from a patient with a morphologically unclassified acute leukemia express IL2-R. These cells were shown to present monoclonal immunoglobulin heavy- and light-chain gene rearrangement. However, Southern analysis of the patient's cellular DNA revealed a germline band in both the J_H and C_k blots. Because Southern analysis was performed on DNA from unseparated MNC containing about 20% residual T cells, the germline configuration could be due to these cells. Alternatively, we cannot exclude that the patient's cells have rearranged heavy- and light-chain immunoglobulin genes on only one chromosome, whereas the other is in the germline. Moreover, the patient's cells had not rearranged TcRβ. In addition, leukemic cells could be induced to differentiate in vitro with PMA into B4+ cells. Taken together these observations indicate the B cell origin of this leukemia.

The myeloid/monocyte-restricted My7 and My9 antigens were expressed on a similar percentage of the patient's leukemic cells as the B cell–specific B4 antigen. The two-color fluorescence studies revealed that 70% of the B4+ cells expressed the My9 antigen whereas 80% of the My9+ cells were also B4+. Moreover, PMA induced expression of B4 and disappearance of My9. These observations suggest that some neoplastic cells may coexpress both myeloid/monocyte and B cell–restricted antigens, as recently reported in acute[29,30] and in hairy cell leukemia.[31] Note that our patient's cells were undifferentiated and did not display myeloid, lymphoid, or hairy cell morphology or lineage-specific cytochemical markers.

Expression of IL2-R by leukemic B cells has been described in hairy cell leukemia[17] and B cell chronic lymphocytic leukemia (B-CLL).[32] In our patient's cells, two-color fluorescence studies showed that 63% of the B4+ cells also expressed the IL2-R. Moreover, the anti-Tac MoAb precipitated a protein of the same mol wt as that of the IL2-R from normal PHA-stimulated T cells, thereby suggesting that the antigen recognized by anti-Tac was indeed the IL2-R.

Fresh leukemic cells did not proliferate in response to rIL2 alone, either in a liquid or a semisolid culture assay, nor did they bind radiolabeled rIL2. However, PHA-LCM, a conditioned medium containing PHA, IL2, and other lymphokines, could induce colony formation from leukemic cells. In addition, preincubation of the blast-enriched cell fraction with PMA allowed, in the presence of rIL2, the generation of large and round B cell colonies. However, because this cell fraction could potentially contain immature T cell precursors, we cannot exclude the possibility that IL2 might act indirectly, ie, bind to an IL2-R–bearing T cell that subsequently could produce lymphokines enhancing the B cell colony growth. Recently, Touw and Löwenberg reported that B-CLL cells could express IL2-R but generated rIL2-induced agar colonies only in the presence of PHA or phorbol esters.[32] Moreover, these authors showed that pre-B leukemic cells, although not expressing IL2-R, can form rIL2-dependent B cell colonies in the presence of PMA.[33] However, Matsuoka et al[34] reported that PMA induced IL2-R expression on patients' immature B leukemic cells but exogenous rIL2 could not induce cell proliferation, thereby suggesting that these IL2-R are not functional. In a recent study we showed that PMA can induce low-affinity IL2-R on the pre-B leukemic cell line Reh-6 (submitted). Incubation of these cells with rIL2 allowed a decreased expression of IL2-R, in contrast to the rIL2-induced upregulation of the IL2-R observed in activated T cells.[35] A similar phenomenon could possibly explain the relatively low proportion of IL2-R–bearing colony cells generated in the presence of rIL2 from PMA-incubated patients' cells (Table 3).

In the present study we could not demonstrate any specific binding of ^{125}I-rIL2 on both fresh and PMA-incubated patients' cells at concentrations lower than 1,000 pmol/L IL2 that detect high-affinity IL2-R.[4] This finding might indicate that the majority, if not all, of the cells express low-affinity IL2-R. However, we cannot exclude the presence of a small number of leukemic cells displaying high-affinity receptors that could not be detected with binding experiments or with a two-day ^{3}HTdR uptake assay but that responded to rIL2 in a colony assay, which reveals only a small number of clonogenic cells. Alternatively, it is possible that the seven-day colony assay may reveal the proliferation of cells bearing low-affinity but functional IL2-R.

An important question raised from previous[17,32] and the present studies concerns the role of the IL2-R expressed on leukemic B cells. Whether IL2 promotes the in vivo proliferation of leukemic B cells and whether it is important for the maintenance of the malignant clone remains as yet unknown.

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Retarded Recovery of Functional T Cell Frequencies in T Cell–Depleted Bone Marrow Transplant Recipients

By John P. Daley, Marta K. Rozans, Brian R. Smith, Steven J. Burakoff, Joel M. Rappeport, and Richard A. Miller

We have studied the effect of removing donor T cells by treatment with the monoclonal antibody Leu-1 and complement before marrow transplantation on the regeneration of functionally competent T lymphocytes in the blood at selected times after transplant. Using sensitive limiting-dilution methods that allow us to enumerate helper, cytotoxic, and proliferating T lymphocyte precursors, we report that regeneration of a functional T cell compartment is more severely impaired for the first 180 days after transplantation in those patients given T cell–depleted bone marrow than in recipients of untreated marrow. After this first 6 months, however, patients given T cell–depleted bone marrow had blood T cell frequencies comparable to those observed in patients given untreated marrow. Diminished frequencies of reactive T cells in recipients of depleted marrow could leave them more susceptible to infection or to the recurrence of neoplastic cells.

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RECOVERY OF PROTECTIVE IMMUNE function after transplantation of allogeneic human bone marrow is typically a protracted process. Blood T cells as assessed by surface antigens usually return to within normal limits within 6 to 12 months after transplantation,[1] but conventional assays of immune function in vitro[2-5] and in vivo[6-8] are frequently depressed for longer periods. Transplant recipients remain abnormally susceptible to infections.[8,9]

On the basis of results of experiments with mouse models,[10] many centers are now attempting to deplete mature T lymphocytes from donor marrow before transplantation in the hopes of preventing the development of graft-*v*-host disease (GvHD).[11-15] Preliminary evidence suggests that T cell depletion can indeed diminish the severity of GvHD, although diminished GvH reactions could pose a risk of increased leukemic recurrence.[16]

We have recently used limiting-dilution (LD) methods to show[17] that most recipients of allogeneic bone marrow exhibit a severe, long-lasting deficit in the proportion of blood T cells that can function in culture. Deficits were demonstrated for helper, cytotoxic, and proliferative function. Many of these patients had normal numbers and subset distributions of blood T cells, and many had normal phytohemaglutinin (PHA) responses in conventional high-density cultures. In this report we use these LD methods to show that recipients of T-depleted marrow have lower proportions of cytotoxic and proliferative T cells for the first 6 months after transplantation than do recipients of nondepleted marrow.

MATERIALS AND METHODS

Patient population and clinical protocol Thirty-eight patients were studied, 11 of whom received T cell–depleted marrow. All patients were advised of the procedures to be used and the attendant risks, in accordance with institutional guidelines, and all gave informed consent Table 1 presents a summary of patient characteristics. A variety of preparative regimens were used, depending on the underlying disorder and clinical condition. The leukemic patients in general received cytosine arabinoside (500 mg/kg/d as a continuous infusion for seven days), cyclophosphamide (60 mg/kg/d for two days), and total body irradiation (either a 1,000-rad midline dose in a single treatment or 1,100 to 1,400 rad in six to eight fractions over three to four days). The patients with aplastic anemia received cyclophosphamide (50 mg/kg/d for four days), procarbazine (12.5 mg/kg/d for three days), and rabbit antithymocyte serum (0.2 mL/kg/d for three days).

Thirty-two patients had HLA-compatible donors, either siblings or, in one case, an HLA-identical father Seven of these HLA-matched individuals received T cell–depleted marrow because they were felt to be at high risk for severe, acute, and/or chronic GvHD in that they were either >30 years old and/or sex mismatched with the prospective donor Four other patients received depleted marrow because they lacked HLA-matched donors. Of these four donors, two were haploidentical (ie, completely matched for one haplotype and mismatched for the other), and two others had identity at one haplotype and partial identity at the other

For those patients who were to receive T cell–depleted marrow, 15 to 20 mL of donor marrow was harvested per kilogram of recipient body weight and the leukocyte fraction concentrated on an IBM Blood Cell Processor. Mononuclear cells were then isolated on Ficoll-Hypaque sedimentation gradients The mononuclear cells were then placed in conical tubes at 20 × 10^6 cells/mL, 25 mL/tube, to which was added 10 μg/mL anti–Leu-1 monoclonal antibody (Becton Dickinson Immunocytometry Systems, Mountain View, CA); after a 30-minute incubation at 4°C, the cells were pelleted and brought up in 25 mL of diluted (1:3) newborn rabbit complement (Pel-Freez Biologicals, Rogers, AR). The cells remained in the complement for 45 minutes at 37°C, were washed once in medium, and in six cases (determined by surviving cell number) were treated with two additional cycles of antibody and complement. After completion of the depletion treatment, the remaining marrow cells were resuspended in 50 mL of medium and infused intravenously into the recipient over 10 to 30 minutes.

It is noteworthy that engraftment, as indicated by recovery of

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Table 1. Summary of Patient Characteristics

	T-Depleted Marrow	Nondepleted Marrow
Number	11	27
Male sex	5	11
Age (mean ± SD)	24 ± 12	17 ± 9
Diagnosis		
Leukemia	10	12
Aplastic anemia	0	7
Other	1	8
AGvHD	3	7
CGvHD	2	13
Disease recurrence	1	0
Died	4	5

Abbreviations: AGvHD, acute GvHD; CGvHD, chronic GvHD.

nonlymphoid hematopoietic cells, was achieved in all recipients of depleted marrow Late graft failure was not observed in any patient. The incidence of chronic GvHD was diminished in this group (see Table 1). Recipients of treated marrow received, on average, 1.0 × 10^8 nucleated donor marrow cells per kilogram of body weight (range, 0.5 to 2.0 × 10^8). T cell depletion was estimated at 99% by flow cytometry (based on Leu-4–positive cells) and at 99% when using the proliferating T lymphocyte precursor (pPTL) assay. The T cell depletion protocol also removes functional helper T lymphocyte precursor (pHTL) and cytotoxic T lymphocyte precursor (pCTL) cells.[18]

Measurement of functional T cell frequencies by LD analysis. Methods to estimate the frequencies of mitogen-responsive precursors for interleukin-2 (IL-2) secreting helper cells (pHTL), proliferating cells (pPTL), and cytotoxic cells (pCTL) have been described in detail elsewhere.[17,18]

Statistical analyses. Because the distribution of LD frequencies within each tested population is nonnormal, simple between-group comparisons (by Student's *t* test) are carried out on log-transformed frequencies, and the resulting geometric means and error factors are presented in the Tables. Some patients had been tested more than once; in these cases we selected the test(s) carried out closest to 6 and 12 months after transplantation for analyses. Step-wise multiple regression analyses to examine the independent effects of age, sex, acute and chronic GvHD, days after transplantation, and marrow depletion on LD results were also performed on log-transformed data.

RESULTS

Delayed regeneration of pCTL and pPTL in recipients of T cell–depleted bone marrow. To see whether T cell depletion from transplanted marrow retarded the recovery of functional T lymphocytes in the blood of bone marrow transplant (BMT) patients, we performed a series of LD tests on 11 individuals who had received marrow treated with anti–Leu-1 antibodies and complement and compared their status to 27 contemporaneous recipients of nondepleted marrow and to healthy young adult values assayed in our lab over the same period of time. We performed independent analyses for data obtained within the first 6 months after transplantation and for data obtained after the first 6-month interval. The results are presented in Fig 1, and summary statistics are shown in Table 2.

We have previously reported[17] that levels of pHTL, pCTL, and pPTL are greatly below normal values for nearly all patients within the first posttransplant year and remain low

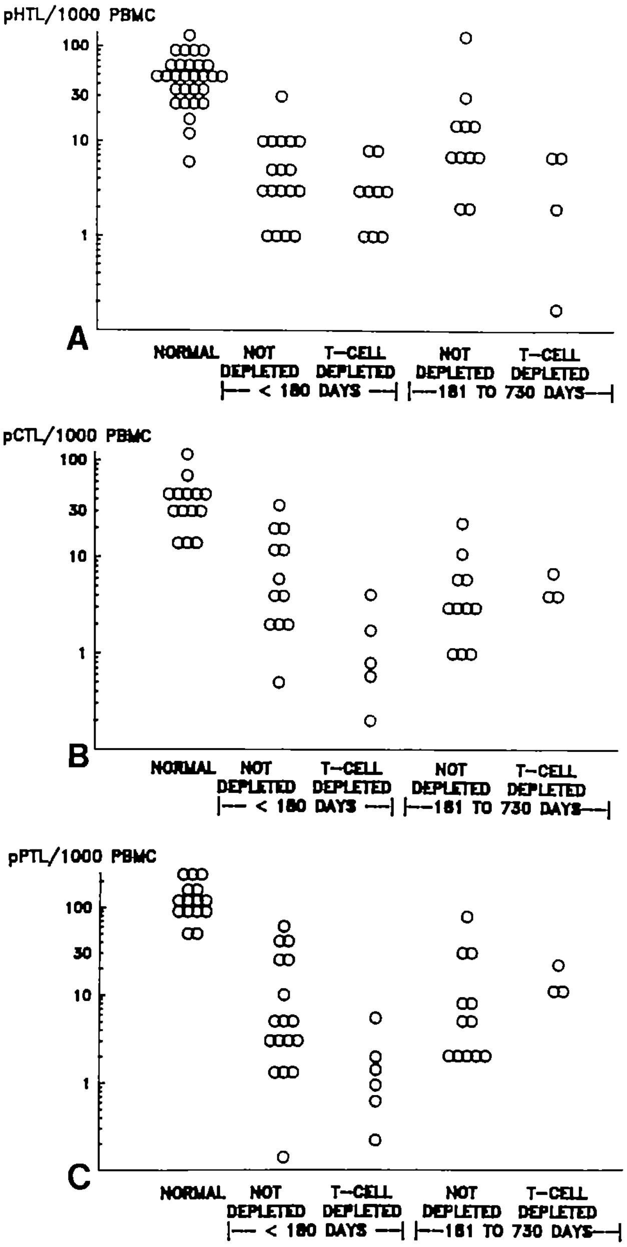

Fig 1. (A), Frequenceis of concanavalin A–reactive precursors for IL-2–secreting helper T cells (pHTL) in the blood of normal controls and BMT recipients at selected times after transplantation expressed as responding cells per 10^3 peripheral blood mononuclear cells (PBMC). (B), Frequencies of PHA/IL-2–reactive pCTL. (C), Frequencies of PHA/IL-2–reactive pPTL.

for most patients for many years after transplantation. The amount of deficit, however, was found (Table 2) to be even more severe for recipients of depleted marrow than for recipients of untreated marrow. Deficits of pHTL, pCTL, and pPTL for control patients were 11-, 7-, and 22-fold compared to normals, respectively, within the first 6 months, but were 20-, 40-, and 100-fold, respectively, for the recipients of T-depleted marrow. The differences between the two groups of transplant recipients could not be attributed to underlying differences in lymphocyte counts (1,107 ± 222

Table 2. Frequency of Functional T Cells in Blood of BMT Patients at Various Times After Transplantation

Treatment	DPT	pHTL*	n	pCTL*	n	pPTL*	n
Normal	—	42.5 (1.1)	29	35.6 (1.2)	14	110.4 (1.1)	15
Nondepleted	<181	3.9 (1.2)	18	5.1 (1.5)	12	5.2 (1.6)	17
Depleted	<181	2.1 (1.3)	9	0.9 (1.6)	5	1.1 (1.6)	6
Nondepleted	181-730	12.8 (1.4)	12	3.9 (1.4)	11	6.5 (1.5)	12
Depleted	181-730	2.0 (5.8)	4	4.6 (1.4)	3	13.9 (1.3)	3

Abbreviation: DPT, days posttransplantation.

*Values presented are the geometric means for *n* patients of the number of pHTL, pCTL, and pPTL present per 1 × 10^3 peripheral blood mononuclear cells. The numbers in parentheses represent the e^{SEM}, the SEM of the ln-transformed data. For data obtained fewer than 181 days post-BMT, the depleted group differs from the nondepleted group in the pHTL, pCTL, and pPTL assays, with Student's *t* test probabilities of .09, .02, and .04, respectively. For data obtained at 181 to 370 days post-BMT, there are no statistically significant differences between depleted and nondepleted groups ($P >$ 1 for all three assays.)

SEM, N = 19, for recipients of undepleted marrow at 91 to 180 days post-BMT as compared with 854 ± 157, N = 11, for recipients of depleted marrow; $P > .1$) but reflect instead differences in the fraction of the blood lymphocytes that responded in culture.

To see whether the effect of marrow treatment could be attributed to differences between the two patient groups in characteristics that could potentially influence T cell regeneration, we performed a step-wise multiple regression analysis to seek independent effects of five such variables in addition to marrow treatment: recipient age, recipient sex, time after transplantation, and the presence of acute or chronic GvHD. In this model, the independent effects of marrow treatment on responder cell frequencies before 6 months post-BMT were found to be significant for pPTL at the level of $P < .03$ and for pCTL at the $P < .001$ level. The trend toward lower pHTL values in recipients of treated marrow was not found to be statistically significant.

The discrepancy in pPTL and pCTL levels between depleted and nondepleted groups diminished in the second half of the first posttransplant year: for tests performed at times more than 6 months after transplantation (Fig 1 and Table 2), there was no significant difference between these two patient populations. Although there is a suggestion, from the limited data available, of continued impairment of pHTL function in recipients of treated marrow, the difference does not reach statistical significance ($P > .05$).

T cell frequencies in the first posttransplant month. It is unclear to what extent functional T cells present in the marrow inoculum contribute to immune function in the transplant recipient during the immediate posttransplant period. To address this question, we have determined precursor frequencies within the first 40 days post-BMT in a small number of patients given T-depleted bone marrow compared with those given untreated marrow. These results for pHTL and pPTL are presented in Table 3; there were too few available pCTL tests within this interval to provide useful information. Although our sample sizes are quite small, precursor frequencies within this early time period seem to mirror those observed at later times within the first 6 posttransplant months (Table 2). Patients given T-depleted marrow had a sevenfold diminution in pPTL within this time period compared with those given untreated marrow ($P < .02$). Helper cell frequencies, though lower in the former patients, were not statistically different from the pHTL frequencies for patients given untreated marrow.

DISCUSSION

The reestablishment of a normally functioning immune system is crucial to the disease-free survival of BMT recipients. We have previously demonstrated[17] that LD methods are extremely sensitive in their ability to detect long-term functional deficits in the helper and cytotoxic T cell compartments of BMT patients. Our present study was undertaken to determine whether the removal of donor T cells from the marrow before transplantation, in an attempt to reduce the incidence of GvHD, had an effect on the level of T cell function seen in the transplant patient in the first 6 months post-BMT. Our findings demonstrate that the level of T cell function (as measured by the pPTL and pCTL tests) within this period is significantly reduced in those patients given T cell–depleted donor bone marrow compared with those given untreated bone marrow. Although we cannot rule out the presence of suppressor cells in the transplant recipients, the linear dose-response curves[17] and the absence of suppressive interactions in mixing experiments (not shown) provided no evidence that the diminished frequencies recorded were due to suppressor cell activity. The LD methods provide only minimal estimates of frequencies, and it is possible that different culture conditions might elicit functional responses from a higher proportion of the cells, but our findings are consistent with and suggest a potential basis for diminished protective immune function in transplant recipients.

How can one account for the especially severe deficit in T cell precursor frequencies seen in recipients of depleted marrow? One possibility is that higher number of immunocompetent T cells seen in recipients of nondepleted marrow

Table 3. Blood T Cell Frequencies in Recipients of Depleted or Nondepleted Marrow: Days 1 to 40 Post-BMT

Marrow Treatment	pHTL	n	pPTL	n
Nondepleted	3.22 (1.7)*	7	5.86 (1.3)†	6
Depleted	1.15 (0.9)	5	0.9 (2.1)	2

*Values presented are the geometric means for *n* patients of the number of pHTL and pPTL present per 1 × 10^3 peripheral blood mononuclear cells. The numbers in parentheses represent e^{SEM}

†Depleted differs from nondepleted at $P < .02$.

might simply represent survival, after transplantation, of T cells present in the marrow inoculum. A rough calculation suggests that this idea is plausible only if the infused cells are all assumed to survive and to restrict themselves to the blood rather than to the peripheral lymphoid system as a whole. Our "average" patient received 10^8 nucleated marrow cells/kg body weight, or 5×10^9/50-kg patient; these 5×10^9 nucleated marrow cells are expected to contain 10^8 pPTL cells.[18] If all of these were to survive and to localize entirely in the blood, they would generate by themselves a pPTL frequency of approximately $10/10^3$ (calculated as 10^8 pPTL/10^{10} total blood lymphocytes), similar to the frequency actually observed in the first 40 days after transplantation (Table 3). If, however, we make the more likely assumptions of incomplete survival and distribution within the 20-fold larger pool of peripheral T cells, the observed blood frequencies fall to <0.5 pPTL/10^3 PBMC, substantially below the observed values.

We suspect, therefore, that most of the pPTL seen in BMT recipients even in the early posttransplant period have developed in the recipients, either from mature T lymphocytes by antigen-driven clonal expansion or from less mature cells. There is evidence that specific humoral immune responses can be transferred from donor to recipient by BMT, but these responses are often transient in the absence of renewed sensitization.[19-21] The development of antiviral responses (determined serologically) to herpes simplex[22] and to cytomegalovirus[23] has been shown not to depend on the pretransplant immune status of the donor, although virus-reactive cells in the transplant recipient are predominantly donor derived.[24] These studies suggest that, although mature, antigen-specific immunocytes can be transferred with marrow to BMT recipients, reestablishment of recipient immune competence depends not on transferred mature cells but on in situ regeneration of new T cells from donor-derived precursors. Whether these precursors are themselves Leu-1+ postthymic T cells and therefore likely to be damaged by the depletion protocol used in our study is unknown.

Differences between recipients of depleted and nondepleted marrow might in addition reflect trophic effects of transferred mature T cells on the development of other, less mature cell types. This would be consistent with delayed recovery of nonlymphoid lineages in recipients of T-depleted marrow.[14,15] An alternate explanation, that the depletion method might inadvertently damage a cell type required for rapid lymphoid recovery, should also be considered despite evidence[13,18] that the method used does not impair function of CFU-GM, CFU-GEMM, or BFU-E.

Finally, the observed differences in T cell function might reflect differences in the two patient groups rather than an effect of depletion per se. For ethical reasons, treatment was restricted to those patients who were thought, on historical grounds, to be at higher risk for development of GvHD; as anticipated, however, the incidence of chronic GvHD was lower in the recipients of depleted marrow. Although there was, in our small sample, no independent, statistically significant correlation between observed precursor frequency and either GvH status or genetic or sex disparity, it would be rash to exclude these factors themselves as potential influences on the pace of T cell regeneration.

Because immunodeficiency after BMT is typically more severe in recipients with GvHD,[8] it is hoped that T cell depletion, by diminishing GvH severity, might lead to improved immune function. Our present findings, however, suggest that the recipients of depleted marrow may, for a limited time, be more severely immunocompromised than patients who receive nondepleted marrow. Follow-up studies using LD methods will be needed to document the late effects of marrow depletion and genetic disparity on regeneration of functional T cells.

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Effects of Granulocyte-Macrophage Colony-Stimulating Factor and Erythropoietin on Leukemic Erythroid Colony Formation in Human Early Erythroblastic Leukemias

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Erythroid colonies from five patients with an early erythroblastic leukemia were obtained in "serum-free" cultures in the presence or absence of recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) and homogeneous native erythropoietin (Epo). Erythroid colonies with abnormal morphology and karyotype could be grown in different culture conditions. Their erythroid nature was ascertained by the presence of carbonic anhydrase I and glycophorin A. Leukemic erythroid progenitors strongly differed from normal progenitors in that spontaneous colonies were always obtained, sometimes with an extremely high plating efficiency (up to 5.7%). Colonies were found to be autonomous from exogenous hematopoietic growth factors because they were still obtained with a high plating efficiency at an average of one cell per culture in the absence of any added growth factor. No evidence for an autocrine secretion of Epo or GM-CSF emerged because Epo or GM-CSF could not be detected by biologic or radioimmunologic assays from the culture supernatant or cellular extracts of the leukemic cells and that Epo or GM-CSF antibodies did not block autonomous growth.

In all cases, however, hematopoietic growth factors increased the plating efficiency of the abnormal erythroid progenitors. In the two "de novo" leukemias, leukemic erythroid progenitors responded primarily to Epo, whereas in the three other patients' (chronic myeloid leukemia) blast crisis they responded maximally to GM-CSF plus Epo. Recombinant erythroid-potentiating activity had no effect in any of these cases. These results suggest that the leukemic erythroid clonogenic cells arise from expansion of erythroid progenitors at different levels of differentiation (ie, CFU-E or BFU-E, depending upon the disease) and that autonomous growth is not related to a secretion of Epo or GM-CSF.

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SEMISOLID CULTURES have clearly demonstrated that the proliferation and differentiation of hematopoietic cells are regulated by distinct growth factors called colony stimulating factors (CSF).[1] Recent progress in protein purification as well as in gene-cloning techniques have permitted the purification of six different human growth factors to homogeneity (ie, macrophage CSF [M-CSF], interleukin 3 [IL3], granulocyte CSF [G-CSF], erythropoietin [Epo], erythroid-potentiating activity [EPA], and GM-CSF[2-8]) and the cloning of their genes. EPA appears to be identical to an inhibitor of metalloproteinase and may not be a true growth factor.[9]

Through the use of these factors, it has been shown that the erythroid progenitors consist of a spectrum of cells ranging from early progenitors that can be grouped together as early and late burst-forming-units (BFU-E) to more mature progenitors called colony-forming-units (CFU-E).[10] The terminal differentiation of early BFU-E is dependent on two different activities, burst-promoting-activity (BPA) and Epo, in contrast to late BFU-E and CFU-E, which appear to be regulated only by Epo.[10] Recent studies with human recombinant GM-CSF, IL3, and G-CSF have shown that, when purified to homogeneity, these factors have BPA.[4,8,11]

Using a polyclonal antibody against isozyme I of carbonic anhydrase (CAI)[12] and the FA6 152 monoclonal antibody (MoAb),[13] we recently diagnosed several cases of early erythroblastic leukemias that phenotypically were compatible with erythroid progenitor cells.[14] This erythroid characterization has been corroborated through ultrastructural studies that have documented ferritin molecules in typical cytoplasmic granules.[15]

In the present study, we have explored the effect of recombinant GM-CSF, recombinant EPA, and homogeneous native Epo on in vitro leukemic erythroid colony formation by using cells derived from five erythroleukemic patients.

MATERIAL AND METHODS

Patients. The first four patients (A, B, C, and D, Table 1) have been described in two previous publications and corresponded to patients no. 5, 6, 7, and 9, respectively, in these manuscripts.[14,15] The fifth patient (E) is a newly diagnosed patient. Briefly, diagnosis of these five cases as erythroid leukemia was based upon the reactivity of the blast cells with an anti-CAI rabbit polyclonal antibody[12] and with the FA6 152 MoAb.[13]

Patients A, B, and C were chronic myeloid leukemia (CML) patients in terminal blast crisis, whereas patients D and E had apparently primitive leukemias. In patient C, the erythroid blasts were mixed in an equal proportion with blasts of the megakaryocytic series.[12,13]

Samples. Peripheral blood from the erythroleukemic patients was collected into sterile syringes containing heparin. Light-density cells were separated over a Ficoll-metrizoate gradient density centrifugation (Lymphoprep, Nyegaard, Oslo)[16] and then washed three

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Table 1. Characteristics of the Five Patients

Patient	Primary Syndrome	Age	Sex	Leukocytes	Blasts (%)	Erythroid Blasts After Ficoll Separation (%)
A	CML	34	M	*	63	90
B	CML	58	F	103,000	19	40-60†
C	CML	17	M	82,000	23	35†
D	—	58	M	52,000	47	80
E	—	62	M	18,000	45	75

*Marrow specimen.

†These percentages were raised after thawing of freezed samples. A variation was observed according to the sample for each patient.

times in Iscove's modified Dulbecco's medium (IMDM, GIBCO, Paisley, Scotland). The cells subsequently were cryopreserved in pure fetal calf serum with 10% dimethylsulfoxide (Lab Bruneau, Paris) by using either a minicooler (Compagnie Française de Produits Oxygénés, Paris) or a standard technique (−20°C for two hours, −70°C overnight) and kept in liquid nitrogen until use.

Marrow cells were obtained from normal marrow donors after informed consent, and an 18-day-old fetal liver was studied after separation of the light-density cells on a Ficoll-metrizoate gradient as before

Semisolid culture procedures Cultures were maintained in "serum-free" medium by using the techniques described by Iscove et al[17] and Steward et al[18] as applied to human cells. Briefly, the medium contained 1 5% deionized bovine serum albumin (Cohn fraction V, Sigma Chemical Co, St Louis),[19] iron-saturated human transferrin (300 μg/mL, Sigma), calcium chloride (28 μg/mL), a mixture of sonicated lipids, 7.5×10^{-4} mol/L α-thioglycerol, 100 ng/mL insulin, and 0.8% 4,000 cp methylcellulose in IMDM. The mixture of lipids was obtained by sonicating 7.8 mg cholesterol, 6.14 mg linoleic acid, and 7.4 mg dipalmitoyl lecithin (all obtained from Sigma) in 10 mL of IMDM (without sodium bicarbonate) containing 1% serum albumin as previously described.[17] Twenty microliters of this mixture was used per milliliter culture. Control cultures were maintained with 10% human AB serum or fetal calf serum. Some experiments were carried out using the plasma clot technique[19] to perform fluorescence labeling of in situ individual colonies.[14]

Biosynthetic human GM-CSF and EPA[5,7] were purified from medium conditioned by Chinese hamster ovary cells, which constitutively express high levels of the biosynthetic proteins from integrated copies of the 91023(B) vector containing the EPA or GM-CSF cDNA EPA and GM-CSF were purified as described previously by using gel filtration and reverse-phase high-pressure liquid chromatography.[20] The protein diluent was used as a negative control in all bioassays. Human urinary Epo was purified to homogeneity by a five-step procedure.[21] Cultures of a patient's cells were usually maintained in a 1-mL volume in a Petri dish (3001, Falcon, Grenoble, France) with cellular concentrations ranging from 10^2 to 10^5 cells/mL. In other experiments, cultures were performed at an average of one cell per well and were initiated in flat-bottomed 96-microwell plates (3072, Falcon) containing 100 μL of serum-free media. Cultures were incubated for eight to 18 days at 37°C in a fully humidified atmosphere with 5% CO_2. Colonies were counted under an inverted microscope.

Secretion of CSF or Epo by leukemic cells was determined by assaying conditioned media or cellular extracts of leukemic cells for the presence of colony-stimulating or Epo activity for normal human marrow of CFU-GM or CFU-E. Conditioned medium was prepared by culturing from 5×10^5 to 1×10^6 leukemic cells/mL in RPMI and 10% fetal calf serum or in IMDM, serum-free conditions from two to 14 days. Conditioned media were recovered and frozen at −80°C until use for culture and for GM or Epo radioimmunoassays Cell extracts were obtained by five rapid cycles of freezing and thawing.

In addition, leukemic cells from patients C and D were incorporated at 1×10^6 cells/mL in a 0.5% agar underlayer. A methylcellulose overlayer containing 5×10^4 normal marrow cells was subsequently added, and colonies were observed at days 7 and 12.

In some experiments, rabbit anti-GM-CSF serum or rabbit anti-Epo serum was directly included in the culture. The GM-CSF and Epo antibodies were obtained by immunizing rabbits with recombinant GM-CSF or homogenous native Epo. A quantity of 5 μL/mL of anti-Epo serum was able to neutralize the activity of 10 IU of exogenous Epo; the anti–GM-CSF was used at a 1/100 dilution. Preimmune serum was used as a control. Because cultures from patient D were inhibited by serum, the anti-Epo IgG fraction was separated from anti-Epo serum by diethylaminoethyl–Affi-Blue gel chromatography (Bio-Rad Laboratories, Richmond, Calif.)

Immunofluorescence studies on cells grown in culture. Cultures grown in methylcellulose with different stimulating factors were individually recovered by adding 1 mL of Hanks' medium to each Petri dish. The cells were washed and counted and their viability evaluated by the trypan blue exclusion test. Subsequently, the cells were cytocentrifuged and fixed with pure methanol for one minute. Double immunofluorescence labeling was performed by first using a pool of three murine anti–glycophorin A (GPA) MoAbs (LICR-LON-R1022, LICR-LON-R18[22] and H85 [Immunotech, Marseille, France]) at a 1/100 dilution followed by a goat $F(ab)'_2$ fragment against mouse Ig conjugated to fluorescein (Bioart, Meudon, France); then the rabbit polyclonal antibody against CAI was applied and revealed by goat IgG against rabbit IgG conjugated to rhodamine. Slides were mounted and observed under a Zeiss microscope equipped with appropriate filters.

The absolute number of CAI-positive cells was calculated by multiplying the total number of cells per culture by the percentage of CAI-positive cells after fluorescence labeling. In all cases, cytocentrifuge preparations were stained with May-Grünwald-Giemsa stain.

When cultures were performed at limiting dilution, some colonies were individually pipetted out of the microwells and transferred to a 0.2-mg/mL polylysine (Sigma)-coated slide, and then fluorescence labeling was performed with the anti-GPA MoAbs. Plasma clot cultures were also labeled in situ by the same antibodies as previously reported.[12]

Karyotypic analysis of the erythroid colonies. Patients A, B, and D were previously cytogenetically characterized[12] Patient C has only a Philadelphia (Ph_1) chromosome. Karyotypic analysis of colonies was performed to distinguish clearly the abnormal erythroid clones from the others. The studies were performed with cells from patients A, B, and D Metaphases from individual colonies were analyzed essentially as described by Dubé et al.[23] Briefly, each culture was monitored for the optimal selection time (ie, day 5 for patient A and day 9 for patients B and D). One hour before harvesting, 0 1 mL of colcemid (GIBCO) diluted to 1 μg/mL was distributed over the culture. Individual colonies were harvested and directly transferred to polylysine-coated slides covered with 2 drops of 0.075 mol/L KCl. After 20 minutes in a humid environment at

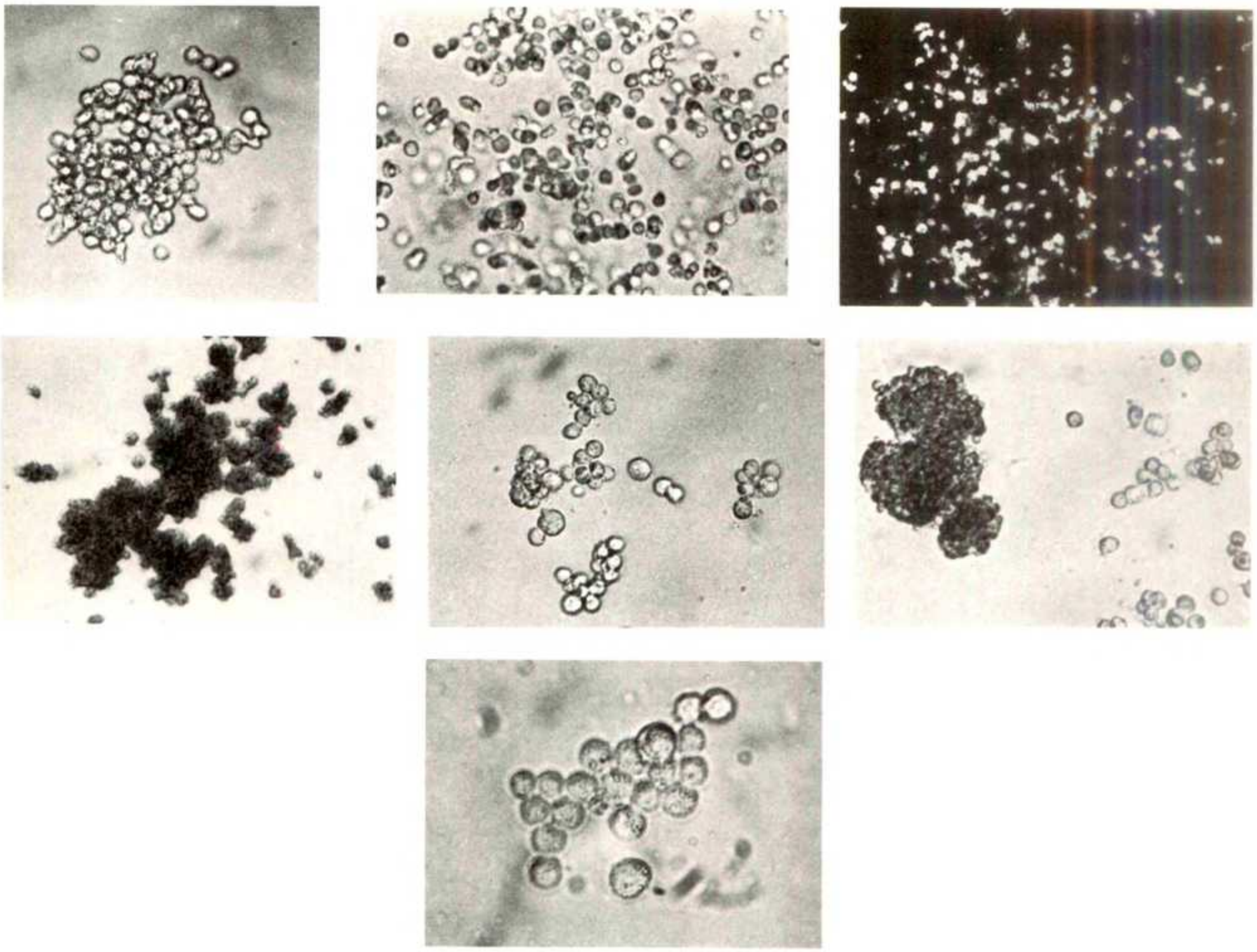

Fig 1. Aspect of abnormal erythroid colonies from the five patients. Cells were grown in serum-free methylcellulose cultures, and colonies were observed under an inverted microscope except for patient B. (A) A colony observed (in patient A) at day 12 after stimulation by GM-CSF (original magnification ×1,400; current magnification ×1,400) (B) A spontaneous colony observed (in patient B) at day 12 of culture (original magnification ×1,400; current magnification ×1,400).(C) A similar colony grown (in patient B) in plasma clot culture and in situ labeled with the polyclonal antibody against CAI after methanol fixation of the culture. Binding was revealed by goat IgG against rabbit IgG conjugated to rhodamine and observed under an episcope at low magnification (original magnification ×700; current magnification ×700). (D) A large erythroid colony grown from patient C in the presence of 100 pmol/L GM-CSF and 1 IU/mL Epo (original magnification ×400; current magnification ×400). (E) A spontaneous colony from patient D (original magnification ×1,400; current magnification ×1,400). (F) An erythroid colony (in patient D) normally hemoglobinized (appearing dark on the photograph) is in close proximity to an abnormal erythroid colony in a culture stimulated by Epo (original magnification ×1,400; current magnification ×1,400). (G) A small colony (in patient E) grown in the presence of Epo (original magnification ×2,800; current magnification ×2,800).

room temperature, the excess hypotonic solution was gently removed, and the slides were fixed with a mixture of methanol:acetic acid (3:1). The slides were then allowed to dry and were Giemsa stained (pH 6.8) to identify the mitoses. R-bands, using giemsa after heating, (RHG) were performed without destaining. All mitoses were examined by using a phase-contrast microscope and photographed. One to seven metaphases per colony were studied.

RESULTS

Characterization of the erythroid colonies. Preliminary results have shown that abnormal erythroid colony formation could be obtained in these five patients under different culture conditions (Fig 1). However, these colonies were atypical in patients A, B, and E, whatever the culture conditions.

In patient A, the colonies were medium sized (50 to 200 cells) and relatively dispersed and maximum at day 12. In patient B, the colonies were large, dispersed, and maximum between days 12 and 18. At this time, a portion of the colonies became much tighter, especially when Epo was added, and they resembled typical BFU-E colonies. In patient E, the colonies were small (up to 50 cells), compact, composed of large cells, and maximum at day 8. The erythroid origin of the colonies from these three patients was established by labeling these cells with erythroid differentiation markers both in a plasma clot, which had the advantage of labeling all colonies in situ, and in methylcellulose cultures. Cells from these colonies exhibited CAI; in addition 40% were labeled by anti-GPA MoAbs. Epo significantly increased the proportion of GPA-positive cells.

In patients C and D, the erythroid colonies were much more typical. A spectrum of erythroid colonies corresponding to early and late BFU-E colonies was present in patient C. In patient D, all the erythroid colonies were similar and looked like late BFU-E. Although more typical in appearance, these colonies were abnormal because they were never as red as the rare colonies with a normal karyotype (Fig 1). In these two patients, cells exhibited both GPA and CAI even in the absence of Epo. This factor markedly increased the terminal maturation of erythroblasts in patient C, as attested by morphological studies.

In patients D and E, only erythroid colonies were observed under a variety of culture conditions. In patients A, B, and C, on the other hand, granulocytic (neutrophilic, basophilic, and eosinophilic) colonies also grew; additionally, a large number of megakaryocytic colonies were seen in patient C even in the absence of a specific stimulating factor. The granulocytic colonies of patients A, B, and C were observed at low cellular concentration only when GM-CSF was added. Mixed erythrogranulocytic colonies were not observed.

Cytogenetic analysis of the erythroid colonies. Cytogenetic studies of bone marrow (patient A) or of unstimulated blood cell cultures (patients B and D) have been reported previously.[12] All metaphases from patient A were hyperdiploid (56 to 64 chromosomes) including a standard Ph_1 chromosome. All metaphases from patient B were abnormal: some had the Ph_1 as the sole abnormality; others had additional changes with a modal chromosomal number of 53. In patient D, all mitoses were abnormal (46 chromosomes), including a marker chromosome resulting from a t(1;15) translocation. Results of the cytogenetic study of colonies are summarized in Table 2. Cells from spontaneous colonies (patients B and D) and from colonies grown in the sole presence of GM-CSF (patient A) showed either hyperdiploid Ph_1-positive karyotypes (except in one colony from patient B) or abnormal karyotypes with the marker chromosome (patient D). The colonies grown in the presence of Epo were hyperdiploid in patient A, either diploid and Ph_1-positive or hyperdiploid and Ph_1-positive in patient B, and either abnormal (with the marker chromosome) or normal in patient D. Dispersed and tight colonies were not cytogenetically distinguishable in patient B.

These results showed that some colonies resulted from blastic cells (hyperdiploid in patients A and B, with a marker in patient D), whereas the others resulted from Ph_1-positive CML chronic-phase cells (granulocytic or erythroid) in patient A and B or from apparently normal cells in patient D (normal karyotype and colonies with a normal appearance).

From this study, it appeared that all colonies from patient A, whatever the culture conditions, belonged to the acute phase of leukemia; in patients B and D, erythroid progenitors from the CML chronic phase or from normal clones were estimated to be less than 5% because these karyotypes were only found in erythroid colonies with a normal aspect.

Regulation of erythroid colony formation by GM-CSF, EPA and Epo in the five patients. In all patients, cultures were first performed on fresh cells, and subsequent studies were repeated on frozen cells in defined medium containing albumin. In patient B, a larger number of spontaneous colonies formed (plating efficiency, 5%) in the absence of exogenous stimulating factor. In patients A, C, and D, "spontaneous" erythroid colonies were present but were less frequent (about 1%), whereas in patient E spontaneous colonies were rare.

Purified recombinant GM-CSF, EPA, and homogeneous native Epo were tested in cultures plated at 1×10^2, 1×10^3, or 1×10^4 cells/mL for patients A to D and 25×10^3 cells to 1×10^5/mL for patient E. A dose-response curve for GM-CSF was performed for patient C; it showed that a 100-pmol/L concentration was optimal for erythroid colonies (Fig 2). In the range 1 pmol/L to 1 nmol/L, no response to EPA was observed (Fig 2). A similar dose-response study was performed for Epo in the presence of 100 pmol/L GM-CSF; a plateau was obtained at 1 IU/mL Epo (Fig 3). In subsequent studies, GM-CSF and EPA were used at 100 pmol/L whereas Epo was added at 1 IU/mL. These three factors were used either alone or in various combinations. Some variations were observed in the absolute plating efficiency of each patient and in the frequency of spontaneous colonies (50% variation). These variabilities were mostly

Table 2. Cytogenetic Studies of Colonies

			Number of Examined Colonies				
Patients	Stimulating Factors	Types of Picked Colonies	Diploid With Ph^1	Hyperdiploid With Ph^1	Mixture Diploid and Hyperdiploid with Ph^1	Normal	Abnormal (Marker)
A	GM-CSF		–	11	–	–	–
	Epo		–	1	–	–	–
B	–	Dispersed	1	21	–	–	–
	Epo	Dispersed	3	2	2 (1 + 6)* (1 + 4)	–	–
	Epo	Tight	4	2	2 (1 + 2)* (2 + 1)	–	–
D	–	Single	–	–	–	–	1
	–	Pooled	–	–	–	–	(18)*
	Epo	Single	–	–	–	1	2
	Epo	Pooled	–	–	–	(1)*	(12)*

Cells were plated in 1 mL Petri dishes in serum-free conditions in the presence or absence of stimulating factor. Colonies were picked as single colonies under an inverted microscope, or colonies were pooled. The karyotype was then studied.

*Numbers in brackets are the numbers of metaphases analyzed.

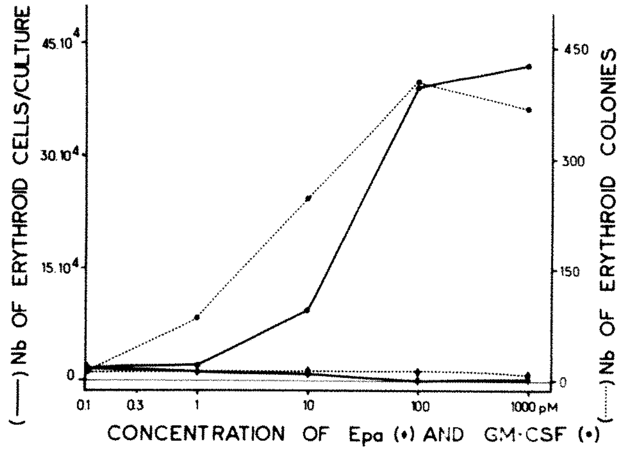

Fig 2. Dose-response curve to recombinant GM-CSF and EPA that illustrates the growth of leukemic erythroid colonies from patient C in the absence of Epo. Cells were cultured in serum-free conditions at 15 × 10^3 cells/mL in triplicate, and colonies were enumerated under an inverted microscope at day 12. Because the maturation of the cells was more complete than in the other patients, colonies were easier to enumerate. It is noteworthy that in this experiment the number of spontaneous colonies was quite low. Cells were subsequently recovered, counted, and cytospinned. Slides were stained by the May-Grünwald staining, and mature and immature erythroblasts were enumerated. The total number of erythroid cells was obtained by multiplying the total number of cells by the frequency of erythroblasts (~50%).

dependent on the data of the sample and of the freezing conditions. However, marked differences were not found in the action of the different growth factors in comparison to fresh cells.

In these patients, neither EPA alone nor EPA combined with Epo and/or GM-CSF had a clear effect on either the number of colonies or on cellular proliferation as expressed in the number of erythroid cells (CAI-positive cells) per culture (Figs 4 and 5). However, in all cases except patient A, Epo clearly enhanced both the plating efficiency and the size of the colonies in comparison to spontaneous colonies. In patient A, Epo significantly increased the number of colonies in comparison to unstimulated cultures in only two of eight experiments. In the remaining six experiments, the number of colonies was slightly but not significantly higher than in the absence of Epo. The results of a typical experiment are represented in Figs 4 and 5. In patients B, D, and E, colonies with a normal appearance were also observed after addition of Epo, but were rare (less than 1% of the morphologically abnormal colonies).

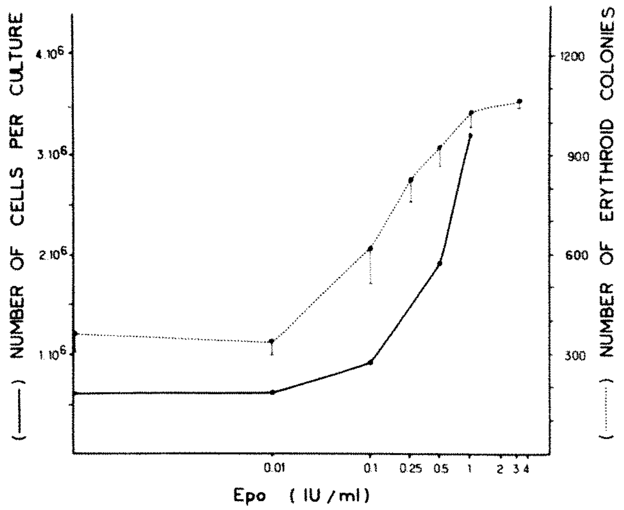

Fig 3. Dose-response curve illustrating the effect of homogeneous Epo in the presence of 100 pmol/L recombinant GM-CSF on the growth of leukemic erythroid colonies from patient C. The experiment was identical to that reported in Fig 2 except that the cells were plated at 1 × 10^4 cells/mL. The frequency of erythroid cells was more than 85% for 1 IU/mL of Epo.

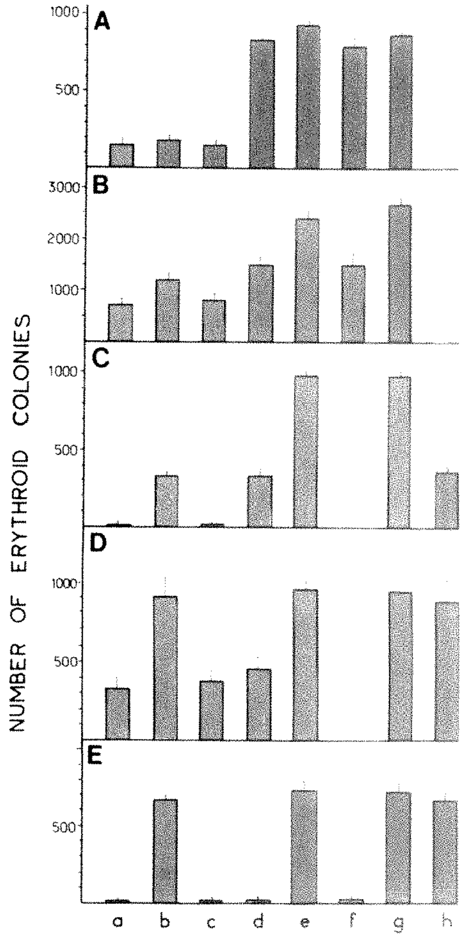

Fig 4. Effect of recombinant GM-CSF, EPA, and homogeneous Epo on leukemic erythroid colony formation from the five patients (A through E). (a) Absence of stimulating factor; (b) 1 IU/mL Epo; (c) 100 pmol/L EPA; (d) 100 pmol/L GM-CSF; (e) 100 pmol/L GM-CSF + 1 IU/mL Epo; (f) 100 pmol/L GM-CSF + 100 pmol/L EPA; (g) 100 pmol/L GM-CSF + 1 IU/mL Epo + 100 pmol/L EPA; (h) 1 IU/mL Epo + 100 pmol/L EPA. Cells were cultured under serum-free conditions; cells were plated in triplicate at 1 × 10^2 cells/mL for patients A and B, at 1 × 10^3 cells/mL for patients C and D, and 1 × 10^4 cells/mL for patient E. Colonies were enumerated at day 12 for all patients except patient D whose colonies were counted at day 8. Results are expressed as the number of erythroid colonies per 1 × 10^4 plated cells except for patient E. In this patient, the number of colonies is indexed to 10^6 plated cells. Column f was not determined in patients C and D; h was not determined in patients A and B.

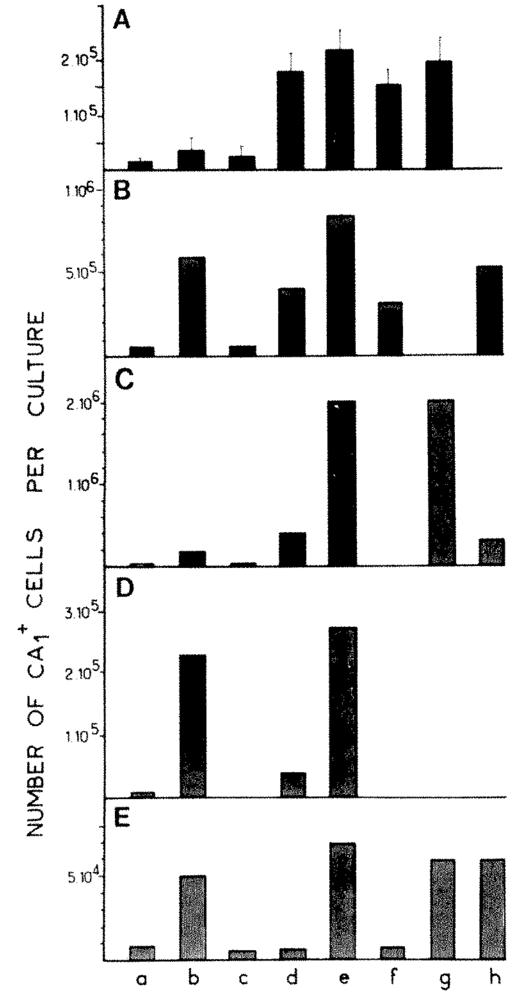

Fig 5. Effect of recombinant GM-CSF, EPA, and homogeneous Epo on the number of erythroid cells per culture in the five patients. (a) Absence of stimulating (b) 1 IU/mL Epo; (c) 100 pmol/L EPA; (d) 100 pmol/L GM-CSF; (e) 100 pmol/L GM-CSF + 1 IU/mL Epo; (f) 100 pmol/L GM-CSF + 100 pmol/L EPA; (g) 100 pmol/L GM-CSF + 1 IU/mL Epo + 100 pmol/L EPA; (h) 1 IU/mL Epo + 100 pmol/L EPA. Experiments were designed as in Fig 4 with the exception that a higher number of cells was plated to recover a sufficient number of cells. Cells were plated at a 1 × 10^4 cell concentration for patients A to D and at 1 × 10^5 for patient E. For patients B to E, results were obtained for one experiment, whereas in patient A they are the average of four independent experiments. Slides were labeled by the anti-CAI antibody revealed by indirect fluorescence after methanol fixation; CAI-positive cells were enumerated under an episcope. The total number of erythroid cells was obtained by multiplying the total number of cells by the frequency of CAI-positive cells. The absence of columns in h for patient A, in g for patient B, in f for patient C, and in e, f, g, and h for patient D means that the experiments were not performed.

GM-CSF alone had a very significant effect in all patients (increasing up to ten times the number of colonies) except patients D and E. The colonies had the same appearance as spontaneous colonies but were larger.

In patients B and C, Epo and GM-CSF acted additively, increasing the number and the size of these colonies, and their combined action gave them an appearance similar to that of normal erythroid colonies. However, they were never frankly red. In patient C, Epo was also added to GM-CSF–stimulated cultures at day 4 of culture, and the plating efficiency was reduced approximately 30% in comparison to GM-CSF–stimulated cultures containing Epo at the onset of the cultures (Fig 6). This loss in the number of colonies was identical to the number of colonies obtained with Epo alone (Fig 6), and the remaining colonies corresponded to the biggest colonies under the optimal conditions.

Therefore, leukemic erythroid progenitors from patients D and E only responded to Epo, whereas those of patients A, B, and C responded maximally to the combination of GM-CSF and Epo. This result was confirmed by studying the relationship between the number of plated cells and the number of colonies. In patients D and E, a linear curve extrapolating to zero defined the relationship between the number of colonies and the number of plated cells when Epo was added alone. A similar curve was found in patient C only when cells were cultured in the presence of GM-CSF plus Epo (data not shown).

The effect of normal human serum and fetal calf serum was tested in all patients. In patients B and E, serum had no significant effect, but in patient C it clearly potentiated the effect of Epo. Surprisingly, the addition of serum markedly inhibited erythroid colony formation in patients A and D. In patient D, serum (both human and fetal calf) at a concentration as low as 40 μL/mL completely inhibited abnormal erythroid colony formation.

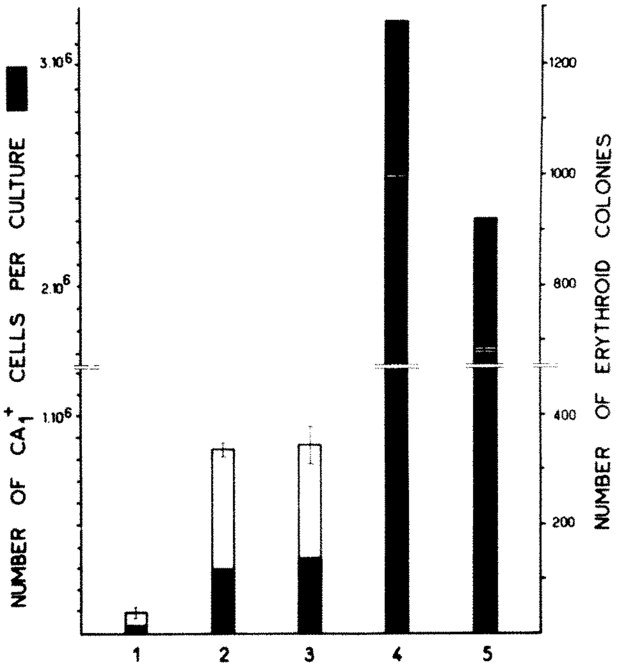

Fig 6. Effect of recombinant GM-CSF and homogeneous Epo on erythroid colony formation and the number of erythroid cells in patient C. (1) Absence of stimulating factor; (2) 1 IU/mL Epo; (3) 100 pmol/L GM-CSF; (4) 100 pmol/L GM-CSF + 1 IU/mL Epo added at the onset of the cultures; (5) 100 pmol/L GM-CSF + 1 IU Epo added at day 4. Cells and colonies were enumerated as in Figs 4 and 5.

Finally, cells from patients were grown at low dilution (average of one cell per well) in individual wells, which resulted in the presence of one cell per well (Table 3). Spontaneous colonies were obtained with a plating efficiency of 1% in patients C and D and 5.7% in patient B. Results paralleled those previously observed in standard cultures. Specifically, progenitors from patient D only responded to Epo, whereas those from patients B and C maximally responded to the combination of Epo and GM-CSF.

Because autonomous growth was observed, we tried to determine whether leukemic cells were secreting a CSF or Epo.

Conditioned medium by leukemic cells or crude cellular extracts were screened for such activities. In none of the patients was a CSF or Epo activity found when using normal human marrow cells as the target. In addition, when leukemic cells from patients C and D were used in an underlayer, they did not promote the growth of CFU-E. Radioimmunoassays for GM-CSF and Epo (kindly performed by Dr E. Goldwasser, University of Chicago) were negative on all conditioned media and cellular extracts.

Anti-Epo serum from 2 to 10 μL/mL was added to the cultures from patients B and C and did not prevent the growth of spontaneous erythroid colonies, whereas it blocked the action of exogenous Epo. In patient D, 5 μL of rabbit serum (immune or preimmune) completely inhibited colony growth. Therefore, rabbit anti-Epo IgG was added to the culture and did not block the spontaneous growth. Moreover, in these three patients, the addition of Epo antibodies did not prevent acquisition of GPA or of Hb by leukemic cells in culture. GM-CSF antiserum was added in culture from patients A and B and did not block spontaneous growth.

DISCUSSION

Using anti-GPA antibodies and earlier markers of erythroid differentiation, we had the opportunity to diagnose ten cases of early erythroid leukemia during an 18-month period. In eight of these, in vitro studies could be performed. In three patients with Down's syndrome, no colony growth could be obtained with any culture procedures (data not shown). In the five patients reported here, leukemic erythroid colony-forming cells could be grown. The leukemic erythroid progenitor cells from the three patients in blast crisis of CML maximally responded to the association of GM-CSF and Epo. In these cases, GM-CSF induced the differentiation and proliferation of the leukemic progenitors, whereas Epo amplified this action and favored terminal differentiation. Therefore, in these three cases, a large part of the leukemic erythroid clonogenic cells behaves as a normal primitive BFU-E.[24,25] In addition, we have presented evidence that a spectrum of leukemic erythroid clonogenic cells was present in two of these patients. Indeed, large and small abnormal colonies bearing the same cytogenetic marker could be grown with GM-CSF plus Epo and with Epo alone, respectively.

Table 3. Plating Efficiency of Leukemic Erythroid Progenitors at an Average of One Cell per Well

	Plating Efficiency (%)			
Patient	0	Epo (1 IU/mL)	GM-CSF (100 pmol/L)	GM-CSF + Epo (100 pmol/L + 1 IU/mL)
B	5.7	10	11	22
C	1	16	10	23
D	1	6	0.5	4

Cells were diluted to 10 cells/mL in serum-free cultures and plated in a 100 μL volume. Six hundred microwells for patient B and 300 microwells for patients C and D were plated without exogenous stimulating factor, whereas 200 microwells were plated in all the other culture conditions.

In the two last patients with de novo leukemias, the erythroid leukemic clonogenic cells only require Epo to form colonies and differentiate. GM-CSF or any conditioned media tested (data not shown) (Mo medium, phytohemagglutinin–leukocyte-conditioned medium, or supernatant from the 5637 cell line) were unable to stimulate growth of these cells, which therefore behave as normal CFU-E or a late BFU-E.

In none of these patients did EPA elicit a response. The action of EPA as a growth factor is actually a matter of debate because it has a homologous structure with a metalloproteinase inhibitor[9] and controversial results have been obtained on the growth of normal erythroid progenitors.[26,27] We are currently investigating the action of EPA on normal cells to explain the absence of response to EPA of leukemic erythroid cells.

The present results are consistent with those previously obtained in acute myeloblastic leukemia (AML). Indeed, a marked heterogeneity in the AML clonogenic cell among different patients was attested to by the surface antigen phenotypes of these cells.[28] In addition, in the same patient an AML cluster-forming cell and a colony-forming cell are present,[29] which suggests the presence of a spectrum of leukemic progenitors. The AML clonogenic cells also respond to recombinant GM-CSF[30]; however, it is not yet known whether their heterogeneity reflects distinctive CSF requirements.

In this study, we could demonstrate that leukemic erythroid progenitors differ from their normal counterparts by a relative independence to growth factors, especially to Epo. In all these patients, more or less spontaneous colonies could be grown that appeared to be really autonomous. Indeed, they were obtained in a serum-free medium that, however, contained albumin and still grew at a very low cellular density, excluding the possibility that their growth was regulated by an accessory cell. Interestingly, karyotypic analysis in a CML patient has shown that in addition to the Ph^1 chromosome all spontaneous colonies except one bear the cytogenetic markers of the blast crisis. This result supports the contention that autonomous growth has been acquired during clonal evolution toward the erythroid blast crisis and was not a growth characteristic of the initial chronic phase.

Spontaneous erythroid colonies have already been described in some human erythroleukemias (M6 of the French-American-British classification and related disorders)[31] and some myeloproliferative diseases (ie in polycythemia vera in which it is a sensitive criterion for diagnosis).[32,33] In this latter disease, it was previously suggested that the spontaneous colonies were hypersensitive to Epo.[34,35] However, a recent study affords strong evidence for their true Epo independency.[36]

In animal models, three different mechanisms have been proposed to explain growth factor–independent proliferation. The first is autocrine secretion of the growth factor[37]; the second is modification of the intracellular transmission pathway of the growth factors,[38] and the third is modification of a growth factor receptor.[39,40]

Previous studies in human AML have shown that leukemic and normal cells have similar growth requirements. This finding led to the suggestion that AML might be a growth factor–dependent rather than an autonomous neoplasm. Recently, Young and Griffin reported three cases of autonomous growth among 15 AML patients. Two of them were related to an autocrine secretion of GM-CSF.[41] The experiments reported here did not afford any evidence for autocrine secretion of a hematopoietic growth factor because neither CSF activity nor Epo activity was detected in conditioned media from leukemic cells. In addition, GM-CSF or Epo antibodies did not block the growth of spontaneous colonies or even the terminal differentiation of leukemic cells. Therefore, these data suggest that one of the two other previously mentioned mechanisms may bypass the growth factor requirement. However, very recently in a few cases of AML, Young et al[42] have reported the presence of GM-CSF transcripts in the absence of detectable active CSF in conditioned media from leukemic cells. Future investigations will have to exclude the possibility that the Epo or other hematopoietic growth factor genes are constitutively expressed in some erythroleukemia without detection of the growth factor itself.

Further studies will be required to determine which mechanism is operating in human erythroleukemia.

Autonomous growth was not the only difference from normal cells. The human erythroleukemic cells also differ by a relative blockage in their terminal differentiation. This blockage is more or less important, depending upon the particular patient, but exogenous Epo facilitated terminal differentiation. Therefore, in erythroleukemia Epo had a differentiation action similar to G-CSF in AML.[4,43] In contrast, GM-CSF did not induce more complete differentiation than the spontaneous one.

Finally, it has to be pointed out that in two cases normal serum from several species could inhibit or abolish the leukemic erythroid growth, which suggests the presence of a powerful inhibitor of this growth in serum. Experiments currently performed in patient D suggest that Transforming Growth Factor-β (TGF-β) is involved in this negative regulation (manuscript in preparation). Serum inhibitor of cell proliferation may also explain the absence of growth of some AML.

In conclusion, in five cases of erythroleukemia we showed that the leukemic erythroid clonogenic cells were the equivalent of CFU-E in two cases of de novo leukemia and BFU-E in three cases of CML blast crisis. These cells differed from normal cells by both an autonomous growth and a relative blockage in their terminal differentiation. The autonomous growth could not be explained by an autocrine secretion of Epo or another hematopoietic growth factor. From this study, it appears that human erythroleukemia can be compared with the avian model. In the chicken, two viral oncogenes act synergistically to cause erythroleukemia. The first one, *v-erb* B, gives the proliferative advantage, whereas the second one, *v-erb* A, blocks the erythroid cell terminal differentiation, thereby giving the phenotype of a CFU-E.[44]

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Autologous Bone Marrow Transplantation Using Unfractionated Cells Cryopreserved in Dimethylsulfoxide and Hydroxyethyl Starch Without Controlled-Rate Freezing

By Patrick J. Stiff, Alan R. Koester, Mary K. Weidner, Kristen Dvorak, and Richard I. Fisher

To develop a simplified method of bone marrow (BM) cryopreservation, changes were made in the standard method in three areas: the cryoprotectant, the method of cell freezing, and the storage temperature. Unfractionated BM cells from 60 patients were cryopreserved in 300-mL aliquots in both dimethylsulfoxide (DMSO) and hydroxyethyl starch (HES), a combination known to preserve granulocytes successfully. The cells were frozen without rate-controlled freezing by simple immersion into a −80°C freezer where they remained until the time of reinfusion. The 60 patients underwent 72 autologous transplants after three high-dose chemotherapy regimens: 30 received high-dose carmustine in combination, five received high-dose busulfan and cyclophosphamide, and 37 received high-dose aziridinylbenzoquinone. The BM was infused for more than 30 minutes after rapid thawing at 37°C. The mean postthaw nucleated cell recovery was 96% ± 11.6%, and Trypan blue dye exclusion was 82.2% ± 9.2%. The mean postthaw CFU-GM and BFU-E recoveries were 81.9% ± 39.0% and 90.5% ± 41.2%. Complete count recovery occurred in 68 of 72 transplants. Median times to a WBC count $>1,000/\mu L$, a granulocyte count $>1,000/\mu L$ and a platelet count $>20,000/\mu L$ were 15, 21, and 15 days, respectively. Risk factors for delayed recovery were not found. Unfractionated BM cells can be successfully cryopreserved in the DMSO/HES mixture rapidly and inexpensively, without rate-controlled freezing or storage at liquid nitrogen temperatures.

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HIGH-DOSE CHEMOTHERAPY with or without total body irradiation, combined with the autotransplantation of cryopreserved bone marrow (BM), is being increasingly used with promising results to treat patients with lymphomas, solid tumors, and even leukemias.[1-9] Methods of BM cryopreservation have been standardized. The cells are frozen in 10% dimethylsulfoxide (DMSO) in a rate-controlled freezer set to cool at a constant 1°C per minute and are then stored until the time of infusion in either the liquid or vapor phase of liquid nitrogen.[1,2,4-6,8,9] To prevent clinical problems due to the severe clumping of granulocytes postthaw, many groups use cell separators to remove them from the BM before freezing, as first described by Weiner et al.[10] Although effective, current cryopreservation techniques are time-consuming as well as expensive, requiring cell separators, rate-controlled freezers, and large liquid nitrogen freezers.

To develop an inexpensive, rapid, yet effective marrow cryopreservation technique we investigated the cryoprotectant combination of DMSO, an intracellular cryoprotectant, and hydroxyethyl starch (HES), an extracellular cryoprotectant that had been previously reported to reduce postthaw granulocyte lysis and clumping[11-13] On the basis of data from previously reported cryopreservation studies, we also questioned the need for rate-controlled freezing[14,15] and storage at liquid nitrogen temperatures.[16-20] In preliminary small-aliquot studies, we showed that the postthaw nucleated cell, and colony-forming unit–granulocyte-macrophage (CFU-GM) recovery of unfractionated BM cells stored in the vapor phase of liquid nitrogen was higher for cells in 5% DMSO and 6% HES than for those in 10% DMSO alone.[21] The postthaw CFU-GM recovery for BM in either DMSO or DMSO/HES was similar to that reported by others using rate-controlled freezing[22-25] despite the fact that the BM in our study was frozen by simple immersion in a −80°C freezer. Additional samples frozen in the DMSO/HES mixture without rate-controlled freezing and stored at −80°C for 12 to 16 months gave a postthaw CFU-GM recovery of 105% ± 39%,[21] which suggests that storage at liquid nitrogen temperatures was also not a requirement for successful BM cryopreservation.

We present here our initial clinical report of a simple cryopreservation technique in 72 autologous transplants done on 60 patients with refractory solid tumors and lymphomas. In this study unfractionated BM cells were cryopreserved in 5% DMSO and 6% HES by simple placement into a −80°C freezer where they were stored until the time of autotransplantation.

MATERIALS AND METHODS

Patients. This study involves 60 consecutive patients who underwent a total of 72 autologous BM transplants between March 1982 and October 1986. Of these, 24 had malignant lymphomas and 36, solid tumors. The median age was 49 (18 to 70), and the male-to-female ratio was 1.3:1. Requirements for entry into the study included an absence of tumor cells in bilateral posterior iliac crest BM aspirates and biopsy samples and normal pelvic x-rays and bone scan results. In addition, the entry WBC, hemoglobin, and platelet counts had to be $>4,000/\mu L$, 10.0 g/dL, and $>150,000/\mu L$, respectively, except for documented cases of anemia of chronic disease. All patients had to provide written informed consent for this institutionally reviewed and approved study.

Cryoprotectant preparation. One hundred thirty-eight milliliters of Normosol-R in 5% dextrose (pH 5.2; Abbott Laboratories, North Chicago, IL) and 42 g of low-molecular weight HES powder (mol wt, 150,000; American Critical Care, McGaw Park IL) were

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placed into a sterile 500-mL bottle. The mixture was autoclaved for 15 minutes to dissolve the HES and sterilize the mixture. After cooling the mixture to room temperature, 100 mL of 25% human serum albumin, used to stabilize granulocyte membranes postthaw,[21] and 70 mL of 50% DMSO (Rimso-50; Research Industries Corp, Salt Lake City) were added. The final volume of the mixture was 350 mL, and the concentrations of DMSO, HES, and human albumin were 10%, 12%, and 8%, respectively. The cryoprotectant was usually made within 24 hours of use. It was stored in glass bottles and kept at 4°C before use.

BM cryopreservation. BM was collected from the anterior and posterior iliac crests and occasionally the sternum as previously described.[26] Cell counts were done intraoperatively to ensure that a minimum of 2 × 10^8 cells/kg body weight were harvested. The cells were mixed with heparinized RPMI 1640 media (M. A. Bioproducts, Walkersville, MD) with a final heparin concentration of 6 to 7 U/mL marrow. No further heparin was added during the processing procedure. After filtering, the entire marrow volume was placed into a single 2,000-mL plasma transfer bag (Fenwal Labs, Deerfield, IL). The subsequent processing was done as a closed-system technique.

To minimize nucleated BM cell loss, the red cells were initially sedimented from the BM by using 6% HES (Hespan; American Critical Care) at 1 vol to 8 vol of BM. The RBCs were allowed to settle for 60 minutes at room temperature. The sedimented RBCs and the cell-rich plasma were each transferred to 600-mL transfer bags and then centrifuged for ten minutes at 400 g at 4°C. The buffy coat cells from the sedimented RBC layer were added to those from the cell-rich plasma and the volume of the combination adjusted to 300 mL with RPMI 1640 tissue culture media. One hundred fifty milliliters of both the cells and the cryoprotectant was simultaneously added to two polyolefin freezing bags (4403-2; Delmed, Canton, MA). The bags were sealed, placed into aluminum freezing frames, and placed horizontally into a −80°C freezer where they remained until reinfusion. Cell counts were recorded before and after the initial processing of the BM and after thawing the cells. Trypan blue dye exclusion testing was done postthaw, and committed stem cell assays were done both before and after the cryopreservation procedure, with the postthaw handling of the BM as previously described.[21]

The cryopreserved BM was thawed at the patients' bedside in a 37°C water bath. Each 300-mL bag was infused through a central venous catheter, without filtering, for more than 30 minutes.

Preparative regimens. The preparative regimens varied depending primarily on the tumor being treated. In 30 cases high-dose carmustine (BCNU; 900 mg/m^2) was used, usually in combination with P16-213 (500 to 750 mg/m^2), cyclophosphamide (4,000 mg/m^2) and cisplatin (100 mg/m^2)[7] or VP16-213 (1,000 mg/m^2) and methotrexate (1,500 mg/m^2). In five cases high-dose busulfan (16 mg/kg) and cyclophosphamide (120 mg/kg) were given as previously described.[27] In 37 cases high-dose aziridinylbenzoquinone (AZQ; 50 to 125 mg/m^2 as a single dose given intravenously over a period of 24 hours) was given. AZQ was being given as a phase I study, with the entry dose being twice the conventional solid tumor dose for this drug.[28]

Committed stem cell assays. CFU-GM and burst-forming unit–erythroid (BFU-E) assays were done precryopreservation and postcryopreservation as previously described with minor modifications[29,30] to assess the efficacy of the freezing technique. For the CFU-GM assays, 1 × 10^5 unfractionated BM cells were cloned in McCoy's media in 1% methylcellulose by using a feeder layer of 1 × 10^6 normal human peripheral blood leukocytes immobilized in 0.5% agar as the source of colony-stimulating activity. The plates were incubated in a 6% CO_2 atmosphere at 37°C. Quadruplicate assays were performed on each specimen, and the plates were counted by using an inverted microscope (Leitz, Rockleigh, NJ). An aggregate of 40 or more cells after ten days in culture was considered to be a colony.

For the BFU-E assays, 1 × 10^5 unfractionated BM cells were cloned in Iscove's media in 1% methylcellulose by using 2 units of sheep Step III erythropoietin (Connaught Laboratories, Swiftwater, PA) per plate. Ten percent phytohemagglutinin-conditioned media was used as the source of burst-promoting factor. The plates were incubated in a 5% CO_2 atmosphere at 37°C. A single aggregate or multiple aggregates consisting of 200 or more red cells after 14 days in culture were considered to be a burst.

The percent postthaw nucleated cell, CFU-GM, and BFU-E recoveries were calculated as percentages of the total number of these cells frozen.

RESULTS

The nucleated BM and stem cell recovery data for the 72 transplants are presented in Table 1. There was only a 9% nucleated cell loss during the initial processing of the unfractionated cells in the 60 harvests. After thawing the 300-mL aliquots there was a negligible cell loss, with approximately 82% of the cells excluding Trypan blue. The BM storage times varied from 1 week to 4 months, although the majority of the transplants were done within the first several weeks after cryopreservation. Prethaw and postthaw CFU-GM and BFU-E assays were available for 63 of 72 and 37 of 72 transplants, respectively. Technical problems with the prefreeze CFU-GM assay prevented the analysis of percent recovery after thawing in nine of the transplants. This was also the case in seven instances for the BFU-E assay. In the remainder, this assay was not attempted prefreeze or postthaw. Percent CFU-GM recovery data from three transplants were censored from the analysis of postthaw recovery because these values were significantly greater than 200% of the pretransplant values.

Table 1. BM Cryopreservation Using 5% DMSO and 6% HES: Nucleated Cell and Committed Stem Cell Recovery During Processing and After Cryopreservation and Mean Harvested/Transplanted Cell Doses

	Recovery (%)	Mean Cell Dose
Nucleated cell recovery during initial processing	91.2 ± 16.7 (60)*	—
Nucleated cell recovery after thawing	96.7 ± 11.6 (71)	—
Trypan blue viability after thawing	82.2 ± 9.2 (65)	—
CFU-GM recovery after thawing	81.8 ± 39.0 (63)	—
BFU-E recovery after thawing	90 5 ± 41.2 (37)	—
Nucleated cells × 10^8/kg harvested	—	2.55 ± 1.00 (60)
Nucleated cells × 10^8/kg transplanted	—	1.94 ± 0 88 (72)
CFU-GM × 10^4/kg transplanted	—	5.4 ± 4.0 (67)
BFU-E × 10^4/kg transplanted	—	5.7 ± 4 1 (44)

Values are expressed as means ± SD.

*Number of harvests or transplants.

Of the 72 BM reinfusions, no clinical toxicity was seen in 45. The most common side effect noted was a transient, slight substernal chest tightness, usually noted at or just after the infusion of the 300-mL aliquots (20.8%). It usually lasted five minutes or less and was not associated with significant dyspnea, cough, fever, nausea, or vomiting. A single episode of nausea and vomiting occurred in ten cases (13.9%), characteristically at the time the patients first experienced the taste of the DMSO. Six patients experienced a mild temperature elevation, usually after the infusion was complete and usually <38°C (8.3%). One patient developed truncal pruritis during the infusion that responded to parenteral diphenhydramine hydrochloride. No abnormalities in the protimes or partial thromboplastin times that were done after the infusions were noted.

The nucleated BM cell and CFU-GM/BFU-E doses transplanted are shown in Table 1. Twelve patients received two transplants each. All were transplanted with $>10^3$ CFU-GM/kg, recently suggested to be a requirement for successful engraftment.[31] The hematopoietic engraftment data are shown in Table 2. Engraftment was considered to have occurred if the total WBC and granulocyte counts were $>1{,}000/\mu L$ and the platelet count remained $>20{,}000/\mu L$ for two consecutive days without transfusions. For all patients, the median time to engraftment was 21 days, with a median of 26 days for the 30 high-dose BCNU transplants, 24 days for the five high-dose busulfan transplants, and 21 days for the 37 high-dose AZQ transplants. Platelet count recovery was more rapid for those receiving high-dose AZQ ($P < .01$) as compared with the others. Although 12 of 37 AZQ transplants were done on patients who had not received any prior myelosuppressive therapy, eliminating these from the analysis did not change the differences in the platelet count recovery ($P < .01$).

There were four deaths before complete engraftment, or 5.6% of the total number of transplants. Two deaths occurred at 12 days posttransplant without any signs of engraftment. One each died of progressive disease and cardiac/noncardiac pulmonary edema with sepsis. The other two died at 24 and 25 days posttransplant of progressive disease and sepsis, respectively. Both had engraftment of granulocytes to $>500/\mu L$. Because three of four of these patients received transplants with $<1.8 \times 10^4$ CFU-GM/kg body weight, we analyzed CFU-GM cell dose as well as several other factors as potential risk factors for slow engraftment. As shown in Table 2, the 12 patients who received transplants with $<1.8 \times 10^4$ CFU-GM/kg body weight as well as those who received transplants with $<1.0 \times 10^8$ nucleated cells/kg and those with a CFU-GM recovery <50% of prefreeze values had an engraftment pattern similar to the entire group.

DISCUSSION

Because of the expense and complexity of the standard BM cryopreservation method, we decided to investigate all aspects of the freezing process. The technique reported here involves significant changes in the standard method in three areas: the cryoprotectant, the method of cell freezing, and the storage temperature of the frozen cells.

Since Lovelock and Bishop first described the cryoprotective effect of DMSO on bovine RBC in 1959[15] it has become the standard cryoprotectant agent for animal[18-20] and human bone marrow cells.[1,2,4-6,8,9,22-24,25,31] Unlike glycerol, another effective agent,[14,16,17,32] DMSO does not need to be diluted or washed from thawed cells, which permits rapid reinfusion after thawing. Although 10% has been established as the optimum concentration of DMSO for the preservation of hematopoietic stem cells,[33] terminally differentiated granulocytes are not successfully preserved. They lyse after thawing and release nucleoprotein and lysosomal enzymes, which causes macroscopic clumping and a gel formation.[22,23] Although many groups remove these cells before freezing to prevent this problem, we decided to investigate the combination of 6% HES with 5% DMSO, which successfully cryopreserves granulocytes.[11-13] Initially reported by Lionetti et al,[11] the recovery of human granulocytes frozen in this mixture ranges from 72% to 101%, with Trypan blue dye exclusion rates of 84% to 89%.[12,13] Our initial in vitro data confirmed the effectiveness of this two-drug mixture for unfractionated BM, with nucleated cell recovery after thawing of 102% ± 13% and Trypan blue dye exclusion of 70% ± 5% for cells stored at −80°C for 12 to 16 months.[21] In addition, we noted no postthaw macroscopic cell clumping or gel formation.

The use of rate-controlled freezing at a constant 1°C/min is based on early studies of Polge and Lovelock[34] and Lewis et

Table 2. Hematopoietic Recovery After Intensive Chemotherapy and the Autotransplantation of BM Cryopreserved in 5% DMSO and 6% HES With Examination of Possible Risk Factors

	Days to				
	Total White Cells	Granulocytes		Platelets	
	$>1{,}000/\mu L$	$>500/\mu L$	$>1{,}000/\mu L$	$>20{,}000/\mu L$	$>50{,}000/\mu L$
All patients (72)*	15	18	21	15	21
High-dose BCNU (30)	16	20	26	22	26
High-dose busulfan and cyclophosphamide (5)	12	11	13	24	35
High-dose AZQ (37)	14	18	21	12†	18
Transplant $<10^8$/kg nucleated cells (8)	16	20	23	12	21
Transplant $<1.8 \times 10^4$/kg of CFU-GM (12)	15	21	25	17	24
Transplants with <50% recovery of CFU-GM postthaw (14)	18	21	15	15	25

Values are expressed as the median time in days to engraft.

*Number of patients receiving transplants.

†$P < .01$ compared with those who received transplants after high dose BCNU and busulfan-cyclophosphamide.

al.[32] The latter group used the colony-forming unit–spleen (CFU-S) assay to assess optimal freezing rates for marrow cells cryopreserved in 12% glycerol. In their study only the duration of the postfreezing plateau and the rate of cooling after the postfreezing plateau affected the CFU-S recovery after thawing.[32] Increasing the postfreeze plateau from 0 to 4 and 0 to 16 minutes, however, decreased the CFU-S recovery only 16% and 28%, respectively. Increasing the cooling rates after the postfreeze plateau from 1 to 3°C/min decreased the CFU-S recovery by only 8%. Other groups have investigated a more rapid freezing technique for human BM cells without a dramatic drop in CFU-GM recovery.[22,35] In fact, Wells et al compared the CFU-GM recovery of unfractionated human marrow cells cryopreserved in 10% DMSO at 1°C/min with those frozen by simple immersion in liquid nitrogen and found that the CFU-GM recovery was 64.8% ± 14.8% for the programmed freezing method *v* 50.6% ± 15.2% for the immersion method.[22] Because of these modest differences and the fact that the cryopreservation of cells by simple placement into a −80°C freezer occurs at a rate of approximately 3°C/min,[11,12,21] we decided to use this as our freezing method. This technique has also been used successfully to freeze platelets[36] as well as granulocytes.[11-13] Thus, although there may be an optimal freezing rate for human BM, there appears to be some flexibility in this rate without causing a severe impairment in the reconstitutive potential of the frozen cells. Our small-aliquot study seemed to verify this.[21] CFU-GM assays done on cells that had been frozen by simple placement into a −80°C freezer and stored for 6 months at liquid nitrogen temperatures showed an excellent postthaw CFU-GM recovery for cells in both 10% DMSO alone (78%) and DMSO/HES (110%).

Early animal studies suggested that the storage of cryopreserved marrow at −79°C was sufficient to give excellent postthaw stem cell growth[17,37] and was able to reconstitute animals after irradiation.[16,17,19,20,33] With the subsequent ability to freeze at liquid nitrogen temperatures, most groups have chosen to store marrow at the lower temperature, with excellent CFU-GM recovery after several years of storage.[24] In reexamining storage at −80°C, we found that for cells stored in the DMSO/HES mixture for periods of 12 to 16 months postthaw CFU-GM recovery was comparable to that of cells stored at liquid nitrogen temperatures.[21]

The results of the present study demonstrate that marrow cryopreservation can be simple, rapid, reproducible, yet inexpensive. The total time to cryopreserve the 700 to 1,200 mL of BM obtained from a typical harvest procedure took as little as two hours, including the 60 minutes needed to sediment the red cells. Cells are concentrated to a volume of 200 mL or less with <10% loss of total nucleated cells. No clumping is seen after thawing, which permits us to freeze the cells in 300-mL aliquots, and there are only minor side effects associated with the infusion of these cells. Most importantly, hematopoietic reconstitution occurred as quickly in our patients as it did for those whose BM cells are cryopreserved in 10% DMSO using rate-controlled freezing and storage at liquid nitrogen temperatures.[1,2,4-6,8]

Although several studies have demonstrated the value of CFU-GM assays in predicting the engraftment of cryopreserved marrow,[25,38] these assays may not necessarily be predictive of pluripotent stem cell numbers. The only proof of true engraftment is the demonstration of hematopoietic reconstitution after lethal marrow damage. Although it is assumed that the busulfan-cyclophosphamide combination used in this study is myeloablative,[27] neither the AZQ nor the BCNU combinations have been definitely shown to be marrow lethal. The BCNU dose used here is, however, a minimum of 50% higher than other recently published studies using this agent in drug combinations with autologous BM rescue.[1,5,8] Although such treatments may not be marrow lethal, autologous transplants do lead to a rapid restoration of blood counts posttransplant, thereby decreasing the potential morbidity, mortality, and the costs associated with the prolonged pancytopenia resulting from such treatments.

We saw no differences in engraftment rates and times among the three preparative regimens used in this study The more rapid platelet count recovery seen in the group receiving AZQ was surprising and appeared not to be due to either prior therapy or dose because patients in the ongoing study receiving as much as 150 mg/m^2 of the drug demonstrated the same phenomena. It is possible that this effect was due to less marrow microenvironmental damage than that caused by BCNU and busulfan.

Although our small-aliquot study demonstrated the viability of CFU-GM after storage for 12 to 16 months at −80°C, the autotransplants done in this study were performed within 4 months of the harvest procedure. Because studies using liquid nitrogen storage temperatures demonstrate that the majority of cell damage occurs during the freezing and thawing of the cells and not the storage time,[24] it is possible that long-term storage at −80°C will be as useful. In an early canine study, the autotransplantation of BM cryopreserved in glycerol and stored at −80°C for 14 months led to rapid reconstitution after lethal irradiation.[39] Longer periods, ie, years, have not been investigated. Studies are in progress to determine whether our small-aliquot data are correct in predicting that hematopoietic reconstitution using cells stored at −80°C for longer than 4 months will be prompt. Although this method can only be currently recommended for the short-term storage of cryopreserved BM, the majority of transplants performed at our and most other centers are done within this time period.

This new method has permitted us to expand our autotransplantation program with a minimal increase in cost and support personnel. It should allow for a wider application of high-dose chemotherapy regimens being developed to treat patients with resistant neoplasms.

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Regulation of Neutrophil Migration and Superoxide Production by Recombinant Tumor Necrosis Factors-α and -β: Comparison to Recombinant Interferon-γ and Interleukin-1α

By Irene S. Figari, Nancy A. Mori, and Michael A. Palladino, Jr

We compared the ability of recombinant human tumor necrosis factor–α (rHuTNF-α) and tumor necrosis factor–β (rHuTNF-β) to stimulate polymorphonuclear neutrophil (PMN) migration and superoxide production. Significant PMN migration occurred across polycarbonate filters after stimulation with rHuTNF-α at concentrations ranging from 10^{-7} to 10^{-10} mol/L and at 10^{-7} to 10^{-8} mol/L for rHuTNF-β and *N*-formylmethionyl-leucyl phenylalanine (FMLP), whereas recombinant human interferon-γ was only minimally active at 10^{-7} mol/L and recombinant human interleukin-1α was inactive at the doses tested. In addition, antibodies to rHuTNF-α completely inhibited rHuTNF-α but not rHuTNF-β or FMLP-induced PMN migration. Combinations of rHuTNF-α and rHuTNF-β (at similar molar concentrations) stimulated PMN migration levels comparable to that obtained with rHuTNF-α alone. Checkerboard analyses performed by placing different concentrations of rHuTNF-α and rHuTNF-β above and below polycarbonate filters of microchemotaxis chambers demonstrated that rHuTNF-α and rHuTNF-β stimulated both chemotactic and chemokinetic responses by PMN. Additional studies demonstrated that 1×10^{-8} mol/L rHuTNF-α and 3×10^{-9} mol/L rHuTNF-β (which represents 10^4 U/mL of each cytokine) were similar in their ability to induce superoxide production by PMNs; however, at ten- to 100-fold lower molar concentrations (10^3 and 10^2 units), rHuTNF-α was significantly more active than rHuTNF-β. At the doses tested, both cytokines were less active than phorbol myristate acetate at stimulating O_2^- release. The results demonstrate that rHuTNF-α and rHuTNF-β differ quantitatively but not qualitatively in their effects on PMN functions in vitro and suggest that rHuTNF-β may be less toxic than rHuTNF-α in vivo.

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TUMOR necrosis factor–α (TNF-α) (also referred to as cachectin) and tumor necrosis factor–β (TNF-β) (also referred to as lymphotoxin) belong to a family of cytokines defined in 1984 that were initially characterized by their ability to induce hemorrhagic necrosis of certain tumors.[1-4] Interferon-γ (IFN-γ) belongs to a family of glycoproteins that has antiviral, immunoregulatory, and antiproliferative functions[5] but does not induce tumor necrosis.[2,5] Recently, TNF-α, TNF-β, and IFN-γ have been shown to exert many similar biologic effects including cytotoxic/cytostatic activities on transformed cell lines; stimulation of HLA-A, -B expression; stimulation of interleukin-1 (IL-1) production; inhibition of lipoprotein lipase activity in adipocytes; and more recently, antiviral activity.[4-15]

It has been reported that recombinant human TNF-α (rHuTNF-α), recombinant human TNF-β (rHuTNF-β), and recombinant human IFN-γ (rHuIFN-γ) stimulate a variety of polymorphonuclear neutrophil (PMN) functions in vitro.[16-26] The finding that rHuTNF-α and rHuTNF-β stimulate these activities is of significant importance because activated PMNs may play an important role in the in vivo activity of TNFs.[17] In contrast, even though natural IL-1 preparations have been shown to be a potent activator of PMNs, recombinant human IL-1α (rHuIL-1α) does not induce tumor necrosis in vivo.[27-30]

The involvement of PMN in inflammatory responses, tumor surveillance, and immune regulation has been suggested.[31-33] Chemokinesis/chemotaxis, a fundamental response of freely moving cells, is believed to be an important process by which circulating leukocytes gain access into a site of tissue damage.[34] Upon interactions with the appropriate stimuli in these sites, leukocytes can produce reactive oxygen species such as superoxide anion (O_2^-) and hydrogen peroxide, which may be involved in local tissue destruction.[31,32]

Because many diseases are characterized by PMN infiltration, we considered it important to compare the relative activities of rHuTNF-α and rHuTNF-β on two PMN functions, stimulation of chemokinesis/chemotaxis and O_2^- release. The results demonstrate that both rHuTNF-α and rHuTNF-β are potent stimulators of PMN migration and O_2^- release. At lower molar concentrations, however, rHuTNF-α is significantly more active than rHuTNF-β in augmenting these functions. rHuIFN-γ only weakly augmented PMN chemotaxis, and in contrast to results obtained with natural IL-1 preparations, rHuIL-1α did not stimulate PMN chemotaxis.[29,30] The results demonstrate that rHuTNF-α and rHuTNF-β differ in their ability to augment PMN functions. The possible roles of activated PMN in the in vivo biologic actions of these molecules are discussed.

MATERIALS AND METHODS

Cytokines rHuTNF-α, rHuTNF-β, and rHuIFN-γ were cloned and expressed in *Escherichia coli* as previously described.[1,2,35] As determined by their cytolytic activities on L-M cells (ATCC CCL1.2; a subclone of NCTC clone 929),[36] rHuTNF-α and rHuTNF-β have specific activities of approximately 5×10^7 and 2×10^8 U/mg, respectively. As determined by an antiviral assay using encephalomyocarditis virus and A549 cells, rHuIFN-γ has an antiviral activity of 2×10^7 U/mg. All preparations were found to contain <0.025 ng endotoxin/mg protein as determined by the limulus amebocyte lysate assay. rHuIL-1α (3×10^6 U/mL) kindly supplied by Dr Peter Lomedico, Hoffmann La Roche, Roche Research Center, Nutley, NJ, contained approximately 150 U/mL of endotoxin as determined by the limulus amebocyte lysate assay.[37]

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Antibodies. Rabbit antibodies to rHuTNF-α were previously described.[38] Briefly, this antiserum has a neutralizing titer of approximately 2 × 10^3 L929 U/μL and does not neutralize rHuTNF-β bioactivity.

PMN isolation. Freshly drawn heparinized blood diluted twofold in 1× Hanks' balanced salt solution (HBSS) was added to an equal volume of 3% dextran T500 solution (Pharmacia Fine Chemicals, Piscataway, NJ). After a 40-minute incubation at room temperature in an upright syringe, the PMN-containing layer was removed and pelleted at 1,200 rpm for five minutes. In some experiments, heparinized blood was separated on Ficoll-Hypaque density gradients before an additional separation on dextran. Residual RBC were lysed by hypotonic shock. The remaining PMN were washed three times in cold 1× HBSS, counted, and adjusted to 8.9 × 10^5 cells/mL in 1× HBSS.

Chemotaxis/chemokinesis measurements. PMN chemotaxis/chemokinesis was measured in multiwell microchemotaxis chambers (Neuro Probe, Cabin John, MD) separated by 5-μm pore size polyvinylpyrrolidone-free polycarbonate membranes.[39] Twenty-five microliters of the desired chemoattractant concentration diluted in HBSS was placed in the bottom chamber. The filter was placed on top, followed by the gasket and the top half of the chambers. Fifty microliters of the PMN suspension (8.9 × 10^5/mL) was added to the upper well and the chambers incubated for one hour at 37°C in 5% CO_2 humidified air. Subsequently, the membranes were removed, fixed, and stained with Diff Quik Staining Kit (American Scientific Products, McGaw Park, IL) and examined microscopically (100× oil immersion) for the number of PMNs that had migrated through the membrane. Five fields were counted and the results expressed as mean cell counts ± SE from replicates of at least three. In each assay, HBSS was used as a negative control, and a positive reference chemoattractant, *N*-formylmethionyl-leucyl-phenylalanine (FMLP, Sigma Chemical Co, St Louis), was used at concentrations from 10^{-7} to 10^{-10} mol/L. In some experiments the chemoattractants were also placed in the upper chambers to distinguish between chemotaxis (directed migration) and chemokinesis (stimulated random migration).

Measurement of O_2^-. O_2^- production by PMNs was measured by the cytochrome c reduction assay as detailed previously.[40] Briefly, PMNs suspended in Earl's balanced salt solution (EBSS) were dispensed in flat-bottom microtiter plates (Falcon No. 3075, Oxnard, CA) at 5 × 10^4 cells/culture. Various concentrations of the chemoattractants prepared in EBSS were added to replicate cultures (four per group). Cytochrome c in EBSS was added (0.2 mg/culture), and the plates were incubated at 37°C in a humidified 5% CO_2 incubator. Thereafter, changes in optical density were measured at various time points of incubation by using an automated enzyme immunoassay reader with a 550-nm filter. Phorbol myristate acetate (PMA), a known stimulant of oxidative metabolism, was added to replicate cultures (25 ng/culture) as a positive control. In each individual case, additional control cultures were performed to demonstrate the inhibition of cytochrome c reduction in the presence of superoxide dismutase (SOD; 30 U/culture). Data expressed as nanomoles of O_2^- production per 5 × 10^4 PMN were calculated as follows: (mean optical density readings/6.3) × 100.

Statistical analysis. Statistics were determined by Student's *t* test.

RESULTS

Stimulation of PMN migration and O_2^- production by rHuTNF-α: comparison with rHuTNF-β. In the initial studies, we compared the abilities of rHuTNF-α, rHuTNF-β, rHuIFN-γ, and rHuIL-1α to induce PMN migration across polycarbonate filters. The results obtained from three individual donors are summarized in Fig 1A. Significant PMN migration was measured over a wide range of rHuTNF-α concentrations ranging from 10^{-7} to 10^{-10} mol/L, which corresponds to approximately 10^5 to 10^2 U rHuTNF-α/mL. The positive chemoattractant FMLP was more active at stimulating chemotaxis at 10^{-6} (data not shown) and 10^{-7} mol/L compared with rHuTNF-α but was less active at the lower concentrations 10^{-8} to 10^{-10} mol/L. At the doses tested, rHuTNF-β was maximally active at 10^{-7} and 10^{-8} mol/L (which corresponds, respectively, to approximately 3 × 10^5 and 3 × 10^4 U rHuTNF-β/mL), and in comparison to rHuTNF-α, rHuTNF-β was only minimally active at 10^{-9} and 10^{-10} mol/L. In contrast, rHuIFN-γ was only minimally active at 10^{-6} mol/L (data not shown) and 10^{-7} mol/L, whereas rHuIL-1α was only minimally active at 10^{-6} mol/L (data not shown) and negative at 10^{-7} mol/L (Fig 1A).

We also compared the ability of rHuTNF-α, rHuTNF-β, and PMA (on a unit-per-milliliter basis) to stimulate O_2^- production by PMN. The results of one of three representa-

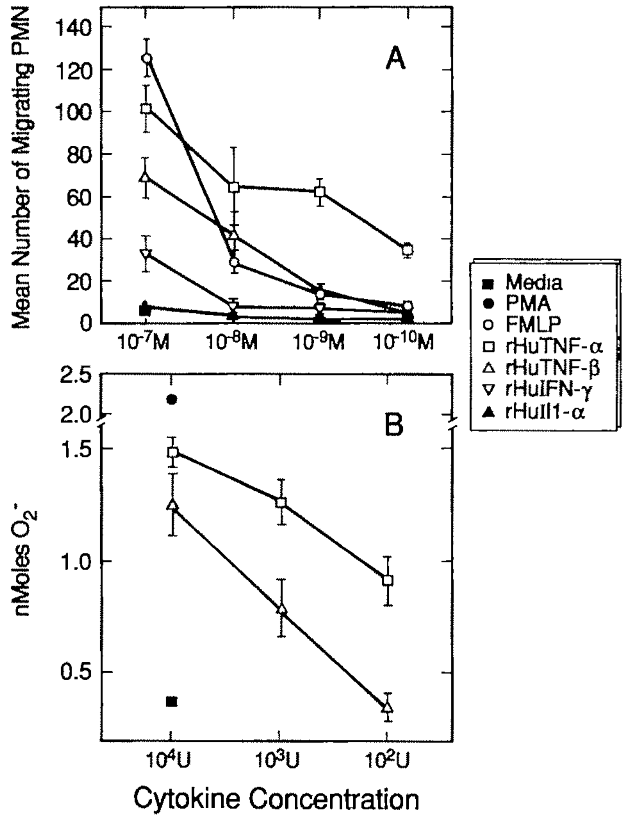

Fig 1. (A) Comparison of cytokine-induced PMN chemotaxis. Results are means ± SE from three individual donors. Statistical analyses demonstrated that rHuTNF-α and rHuTNF-β were similar in their ability to induce chemotaxis at 10^{-7} and 10^{-8} mol/L (P is not significant). However at 10^{-9} and 10^{-10} mol/L, rHuTNF-α was significantly more active ($P < .02$ and $P < .01$ respectively). (B) Comparison of TNF-induced O_2^- production by PMN. Results are means ± SE from four replicates. Statistical analyses demonstrated that rHuTNF-α and rHuTNF-β were similar in their ability to induce O_2^- release at 10^4 U/mL (P is not significant). However at 10^3 and 10^2 U/mL, rHuTNF-α was significantly more active ($P < .05$ and $P < .01$ respectively).

Table 1. Assessment of Chemokinetic and Chemotactic Responses of Human PMN to rHuTNF-α and rHuTNF-β

		rHuTNF-α/rHuTNF-β in Upper Chamber (Mean Number of Migrating PMN ± SE at)				
Experiment No.	Lower Chamber	HBSS	10^{-7} mol/L	10^{-8} mol/L	10^{-9} mol/L	10^{-10} mol/L
rHuTNF-α						
1A	HBSS	3 ± 1	62 ± 14	97 ± 9	38 ± 3	24 ± 4
	10^{-7} mol/L	91 ± 5	32 ± 7	63 ± 7	73 ± 1	106 ± 6
	10^{-8} mol/L	88 ± 7	34 ± 11	71 ± 1	38 ± 3	53 ± 9
	10^{-9} mol/L	57 ± 11	61 ± 6	53 ± 6	52 ± 7	66 ± 11
	10^{-10} mol/L	6 ± 3	59 ± 4	49 ± 11	17 ± 1	23 ± 6
rHuTNF-β						
1B	HBSS	9 ± 1	8 ± 3	74 ± 4	37 ± 5	
	10^{-7} mol/L	125 ± 14	36 ± 5	118 ± 9	68 ± 5	
	10^{-8} mol/L	77 ± 3	22 ± 3	101 ± 11	49 ± 7	
	10^{-9} mol/L	19 ± 5	16 ± 3	57 ± 5	58 ± 4	

Chemotaxis is shown on the vertical axis and chemokinesis along the diagonal axis.

tive experiments illustrated in Fig 1B show that both rHuTNF-α and rHuTNF-β can stimulate SOD-inhibitable O_2^- release by PMN. Similar to the results obtained with chemotaxis, rHuTNF-α was significantly more active at inducing O_2^- release than rHuTNF-β at lower concentrations (10^3 and 10^2 units), but at 10^4 U/mL, the highest concentration tested (which corresponds to 1×10^{-8} mol/L for rHuTNF-α and 3×10^{-9} mol/L for rHuTNF-β), both cytokines showed similar activity. At the concentrations tested, both cytokines were less active than PMA at inducing O_2^- release.

To further define the response of PMN to rHuTNF-α and rHuTNF-β, we attempted to determine whether migration across polycarbonate filters required a concentration gradient. A series of checkerboard analyses were performed, and one of four representative experiments is shown in Table 1. As shown, stimulation of PMN migration by rHuTNF-α (experiment 1A) or rHuTNF-β (experiment 1B) involved both chemotactic and chemokinetic responses. Maximal induction of PMN migration did not require a positive concentration gradient between the lower and upper chambers because the presence of a negative gradient (created by placing the higher rHuTNF-α or rHuTNF-β concentrations in the upper chamber) also enhanced PMN migration. However, a chemotactic response was noted for both rHuTNF-α and rHuTNF-β at 10^{-7} mol/L, which suggests that these agents induce both chemotactic and chemokinetic responses of PMN. Polyclonal antibodies to rHuTNF-α abolished rHuTNF-α but not rHuTNF-β or FMLP-induced PMN migration, thereby indicating that the PMN response was not induced by contaminants in the recombinant preparations (Table 2).

Table 2. Effects of Anti-rHuTNF-α on Chemotactic Activity of rHuTNF-α, rHuTNF-β, and FMLP

	Treatment (Mean Number of Migrating PMN ± SE)	
Chemoattractant	HBSS	Anti-rHuTNF-α*
10^{-9} mol/L rHuTNF-α	88 ± 1	0 ± 0
10^{-8} mol/L rHuTNF-β	57 ± 2	66 ± 5
10^{-8} mol/L FMLP	28 ± 4	40 ± 8

*Added at a concentration sufficient to neutralize a fivefold excess of rHuTNF-α.

TNF binding studies. Previous studies have demonstrated that both rHuTNF-α and rHuTNF-β bind to similar receptors on PMN.[19,41] Additional studies were therefore performed to compare the relative abilities of these cytokines to compete for their receptors as measured by changes in PMN migration. As shown in Fig 2, combinations of similar molar concentrations of rHuTNF-α and rHuTNF-β stimulated PMN migration levels comparable to that of rHuTNF-α alone, which suggests that the relative binding efficiency of rHuTNF-α for its receptors on PMNs is greater than that of rHuTNF-β.

DISCUSSION

Studies describing a chemotactic response of various lymphoid populations to rHuTNF-α have been recently reported.[42,43] The results presented in this study also demonstrate that, depending on the dose of rHuTNF-α or rHuTNF-β used either a chemotactic or chemokinetic response can be obtained, which suggests that PMN infiltration into a site of inflammation may be independent of a concentration gradient of locally released rHuTNF-α. Although the enhancement of PMN migration appears to be a function of multiple cytokines, the results presented indi-

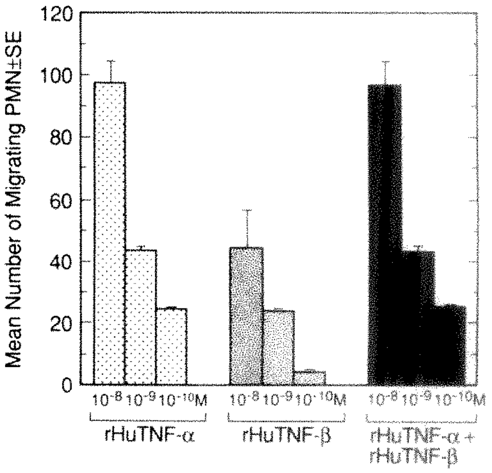

Fig 2. Combined effects of rHuTNF-α and rHuTNF-β on PMN chemotaxis. Results are means ± SE of triplicate determinations.

cate that both quantitative and qualitative differences exist in their ability to stimulate this function. Although rHuTNF-α induced significant PMN migration from 10^{-7} to 10^{-10} mol/L and rHuTNF-β did from 10^{-7} to 10^{-8} mol/L, rHuINF-γ was only minimally active at 10^{-7} mol/L and negative at 10^{-8} to 10^{-10} mol/L. In support of these results are previous studies that have demonstrated that recombinant bovine IFN-γ does not induce significant PMN chemotaxis, although crude conditioned media obtained from bovine mononuclear cells stimulated with infectious bovine rhinovirus (possibly because of TNF-α and/or TNF-β contamination) does.[23] In contrast to previous results with natural IL-1, our data indicate that at 10^{-7} to 10^{-10} mol/L rHuIL-1α does not induce PMN migration, which suggests that the natural IL-1 preparations may have been contaminated with TNF-α and/or TNF-β.[29,30]

The simultaneous treatment of PMN with both rHuTNF-α and rHuTNF-β induced PMN migration comparable to that obtained with rHuTNF-α alone, thereby supporting earlier studies that both factors bind to similar or closely related receptors and indicating that the relative binding efficiency of rHuTNF-α for its receptor on PMN is significantly greater than that of rHuTNF-β. This difference in binding efficiency may therefore explain the quantitative differences in activities of these agents.[41] In support of these observations are our recent results demonstrating that rHuTNF-α also exerts greater cytolytic activity against ME180 cervical carcinoma cells compared with rHuTNF-β (at the same concentration) and that the combination of both agents (Figari and Palladino, unpublished results) results in a cytolytic activity comparable to that of rHuTNF-α alone.

Stimulation of PMN migration by rHuTNF-α and rHuTNF-β to a site of inflammation may play an important role in the events leading to hemorrhagic necrosis in vivo[27,44,45] This is supported by our observations with mice bearing established subcutaneous Meth A sarcoma implants that rHuTNF-α and rHuTNF-β but not recombinant murine IFN-γ or rHuIL-1α (*a*) induce hemorrhagic tumor necrosis, (*b*) exert significant antitumor actions, and (*c*) induce moderate infiltration of leukocytes into the tumor site.[27] Similar results have also been reported after treatment of Meth A sarcoma–bearing mice with endotoxin,[44] which suggests that hemorrhagic tumor necrosis is a type of local Shwartzman reaction.[27,44,45]

It has been reported that different leukocyte populations secrete specific chemoattractants.[46-52] More recently, studies have described a factor released by natural killer cells that activated monocyte oxidative metabolism as well as stimulated PMN chemotaxis.[53] Although the nature of these factors is not known, it appears likely that the chemotactic factor from natural killer cells and mast cells is TNF-α because recent studies have demonstrated that purified large granular lymphocytes and IL-3–dependent mast cell lines produce TNF-α but not TNF-β.[54-57] In addition, the factor from other leukocyte populations may represent either TNF-β or other as yet undefined products.

Increased PMN adherence to vascular endothelium, a prerequisite for extravascular migration of PMN, may be critical for induction of vascular injury associated with inflammatory reactions.[32] rHuTNF-α has been shown to enhance the expression of proteins on endothelial cells required for leukocyte adherence.[58,59] In addition, we have recently demonstrated that PMNs activated by rHuTNF-α cause disruption and suppression of endothelial cell proliferation.[19] TNF therefore may function by two mechanisms: (*a*) directly by activating PMNs to alter endothelial cell integrity by releasing IL-1,[59] activated oxygen species,[16-18,20] and other factors[25]; or (*b*) directly on the endothelial cells to release procoagulant activity and/or IL-1.[10,60,61]

The rHuTNF-β used in these studies was produced in *E coli* and was nonglycosylated whereas native TNF-β is glycosylated. It is possible therefore that the absence of carbohydrate moieties explains the quantitative differences in activity between rHuTNF-α and rHuTNF-β. However, because rHuTNF-α and rHuTNF-β bind to the same or closely related receptor, it is unlikely that the carbohydrate residues are in the area of the rHuTNF-β molecule responsible for receptor binding.[41] In support of our data are recent results describing the disparate effects of rHuTNF-α and rHuTNF-β on (*a*) hematopoietic growth factor production and neutrophil adhesion molecule expression by cultured human endothelial cells and (*b*) increasing plasma fibrinogen, a marker of liver acute-phase inflammatory responses.[62,63] However, recent studies have demonstrated similar abilities of rHuTNF-α and rHuTNF-β to induce IL-1 production by both human peripheral blood mononuclear cells (Palladino, and Figari, unpublished observations) and endothelial cells (Pober, personal communications). Of interest, the rHuTNF-β used by Pober et al was glycosylated. Moreover, when using the L-M bioassay, rHuTNF-β is more active in vitro than rHuTNF-α.[36]

In conclusion, the data demonstrate quantitative but not qualitative differences in the ability of rHuTNF-α and rHuTNF-β to induce PMN migration and O_2^- production, which may be related in part to differences in their relative binding efficiencies for a specific receptor(s) on the surface of lymphoid and endothelial cells.[19] Further studies are needed to characterize the roles of TNF in regulating the interactions between PMNs, endothelial cells, and the resulting inflammatory reactions.

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The von Willebrand Factor Domain–Mediating Botrocetin-Induced Binding to Glycoprotein IB Lies Between Val$_{449}$ and Lys$_{728}$

By Yoshihiro Fujimura, Linda Z. Holland, Zaverio M. Ruggeri, and Theodore S. Zimmerman

Botrocetin, a component of *Bothrops jararaca* venom, induces von Willebrand factor (vWF)-dependent platelet agglutination and has been proposed as an alternative agent to ristocetin for evaluating vWF function. However, important differences between the vWF-platelet interactions induced by these two agents have suggested that different regions of vWF and the platelet may be involved in the interactions induced by the two agonists. We have recently demonstrated that binding of vWF to the platelet glycoprotein (GP) Ib receptor, either induced by ristocetin or as occurs spontaneously with asialo-vWF or vWF from IIB von Willebrand disease, is mediated by a domain residing on a 52/48-kilodalton (kD) tryptic fragment of vWF. This fragment extends from amino acid residue Val (449) to Lys (728). We have now found that this 52/48-kD fragment blocks botrocetin-induced binding of vWF to platelets and completely inhibits botrocetin-induced platelet agglutination. These results provide evidence that the vWF domain–mediating, botrocetin-induced platelet agglutination lies within the region delimited by this fragment and is therefore close to or identical with that which mediates ristocetin-induced binding and spontaneous binding of vWF to platelet GPIb. Anti-GPIb monoclonal antibodies also blocked agglutination, which showed that botrocetin, like ristocetin, induces binding of vWF to the GPIb receptor.

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VON WILLEBRAND FACTOR (vWF) plays an essential role in primary hemostasis and circulates in blood as a series of multimers composed of a 2,050–amino acid residue subunit.[1,2] The ability of vWF to support ristocetin-induced platelet agglutination has been widely used to assess vWF function in vitro.[3]

Read et al and Brinkhous and Read[4,5] have found that botrocetin, a component of venom from the species *Bothrops jararaca*, causes vWF-dependent platelet agglutination. They suggested that botrocetin could substitute for ristocetin in an assay of vWF function.[5] However, several important differences between these two agonists have been noted. Plasma from patients with type IIA vWD lacks the largest vWF multimers and has markedly decreased levels of ristocetin cofactor activity. However, the botrocetin cofactor activity is normal in such plasma.[6,7] Botrocetin can function in the presence of negatively charged heparin[4] or the ristocetin analogue vancomycin,[7] whereas ristocetin does not. Platelet-rich plasma (PRP) from patients with Bernard-Soulier syndrome, in which qualitative and quantitative abnormalities of platelet glycoprotein Ib (GPIb) are noted, shows partial platelet aggregation with botrocetin but a total lack of platelet agglutination by ristocetin.[7] Based on these observations, Howard et al have postulated that different functional sites on both the platelet and vWF molecule are involved in interactions induced by ristocetin and botrocetin.[7]

Recently we have determined that the GPIb-binding domain of human vWF resides on a tryptic fragment that extends from amino acid residue 449 to 728 of the vWF subunit.[8,9] We have demonstrated that this fragment directly binds to the GPIb receptor without any platelet agonist and independently of platelet metabolism.[8,10] In this paper we present evidence that the botrocetin-induced vWF binding to platelets is mediated by a domain that also lies between amino acid residues 449 to 728 and is close to if not identical with that mediating ristocetin-inducing binding. We also show that binding is to the GPIb receptor.

MATERIALS AND METHODS

Carrier-free Na [^{125}I] was obtained from Amersham Corp (Arlington Heights, IL). Ristocetin, apyrase, trypsin (bovine pancreatic type I, 11,300 to 15,000 U/mg), and *B jararaca* venom were purchased from Sigma Chemical Co (St Louis). Crystallized bovine serum albumin (BSA) was obtained from Calbiochem-Behring Corp (La Jolla, CA). Leupeptin was supplied by Chemicon (El Segundo, CA). Iodogen was from Pierce Chemical Co (Rockford, IL) Other reagents were of the best grade commercially available.

Purification of botrocetin Venom coagglutinin (botrocetin) was purified from *B jararaca* venom with modifications of the method of Read et al[4] as follows: crude venom was dissolved in 0.02 mol/L Tris-HCl 0.1 mol/L NaCl buffer, pH 7.35, then applied to a high-performance liquid chromatography (HPLC) anion-exchange column (Mono Q column; Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with the same buffer. After extensive washing, the column was eluted with a linear salt gradient (0.1 to 0.7 mol/L NaCl) at 1.0 mL/min for 30 minutes. Fractions that contained platelet coagglutinin activity but were devoid of thrombinlike activity were pooled and dialyzed against 0.05 mol/L Tris-HCl–0.15 mol/L NaCl buffer, pH 7.3 (TBS), at 4°C overnight and then frozen at −70°C until used.

Preparation of highly purified vWF. Highly purified vWF was isolated from cryoprecipitate (the generous gift of the American Red Cross, Bethesda, MD) as previously described.[11]

Radiolabeling of proteins. [^{125}I] radiolabeling was performed with the Iodogen method [12] Free [^{125}I] was removed by a disposable Sephadex G-25 column (PD-10, Pharmacia). The labeled vWF preparations had a specific activity of 0.28 to 0.92 mCi/mg.

Trypsin cleavage of vWF and separation of fragments. The methods used have been recently reported in detail.[8] Trypsin digestion was performed by mixing 1 mg of vWF with 2,500 units of

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enzyme and incubating for two hours at 37°C, pH 7.0. The fragments generated were separated by HPLC size exclusion into six main fractions as described.[8]

The isolation of the 52/48-kilodalton (kD) doublet as well as other fragments of 13 kD, 22 kD, 41 kD, and 55 kD was performed by HPLC chromatofocusing and size exclusion chromatography in the presence of 6 mol/L urea after reduction and *S*-carboxymethylation of each fraction.[8] All purified tryptic polypeptides were dialyzed against TBS, pH 7.3, and stored at −70°C until use.

Monoclonal antibodies. Murine monoclonal antibodies to GPIb were AP1 (the generous gift of Dr Robert Montgomery, Milwaukee), LJ-Ib1, and LJ-Ib12, which have been described and characterized previously.[13,14] Murine monoclonal antibodies to GPIIb/IIIa were LJ-P5 and LJ-CP8, which have also been described previously.[15,16] IgGs were purified from ascites fluid by protein A–Sepharose CL-6B (Pharmacia) affinity chromatography according to Ey et al.[17]

Preparation of washed, gel-filtered platelets and formalin-fixed platelets. PRP from fresh acid-citrate-dextrose–blood from normal volunteers was obtained as described.[8] Apyrase was added to the PRP at a final concentration of 5 adenosine triphosphatase U/mL. Washed, gel-filtered platelets were prepared by a combination of BSA density gradient centrifugation[18] and Sepharose CL-2B gel filtration[19] as described.[8] Washed, gel-filtered platelets were suspended in calcium-free Tyrode's buffer containing 0.1% BSA, pH 7.3.

Formalin-fixed platelets were prepared by overnight fixation of gel-filtered platelets with 1% formalin.[20] Formalin-fixed platelets were washed three times with TBS, suspended in TBS, pH 7.3, containing 0.02% NaN_3 and 10 μg/mL leupeptin, and stored at 4°C.

Botrocetin-induced binding of [^{125}I]vWF to formalin-fixed platelets. Assays were performed in an incubation volume of 125 μL in plastic Eppendorf tubes. In most experiments, [^{125}I]vWF, formalin-fixed platelets, and botrocetin were added to the tubes at final concentrations of 5 μg/mL, 10^8/mL, and 5 μg/mL, respectively. For competitive inhibition studies, various ligands dissolved in TBS, pH 7.3, were added to the incubation mixture at concentrations described in Results. After 30 minutes' incubation at room temperature, two 50-μL aliquots of the mixture were layered over 20% sucrose cushions (300 μL) in 500-μL Sarstedt microfuge tubes (Princeton, NJ), and bound ligand was separated from free ligand by a four-minute centrifugation at 12,000 g in a Beckman Microfuge B (Palo Alto, CA). The tube tips containing bound ligand were amputated and counted in a gamma counter. Nonspecific and nonsaturable binding, determined in the presence of a 50-fold excess of unlabeled vWF added together with the labeled ligand, was less than 15% of total binding. Specific binding was calculated by subtracting nonspecific binding from the total. The 50% inhibition constant (IC_{50}) was that concentration of competing ligand that inhibited the specific binding of [^{125}I]vWF by 50%.

Platelet aggregation studies. They carried out at a final platelet concentration of 3 to 3.5 × 10^8/mL in a Lumi aggregometer (Chrono-Log Corp, Havertown, PA) with 0.3 to 0.5 mL of washed, gel-filtered platelets or formalin-fixed platelets.

Protein concentration. The concentration of purified monoclonal IgG was calculated from absorbence at 280 nm and an extinction coefficient of $E^{1\%}_{280}$ = 14.3. Concentrations of other proteins were measured by the method of Bradford[21] with BSA as a standard.

RESULTS

Botrocetin-induced binding of vWF to platelets. The concentrations of botrocetin that induced maximum vWF binding to platelets were determined at room temperature by adding increasing amounts of botrocetin to a mixture containing fixed amounts of [^{125}I]vWF (5 μg/mL) and formalin-fixed platelets (10^8/mL). Maximum binding occurred between 2 and 10 μg/mL of botrocetin. At concentrations higher than 10 μg/mL the binding was reduced (not shown). Thus, a concentration of 5 μg/mL of botrocetin was chosen for binding assays.

[^{125}I]vWF binding to formalin-fixed platelets in the presence of botrocetin was measured at 4°C, 23°C, and 37°C. The binding was almost maximum after 15 minutes' incubation, and there were no differences in the extent and rate of [^{125}I]vWF binding at the different temperatures (not shown). Therefore, all the succeeding experiments were performed at room temperature with 30 minutes' incubation time.

To determine when the binding was saturable, [^{125}I]vWF (3 to 197 μg/mL) was incubated with formalin-fixed platelets in the presence of botrocetin (Fig 1). The data were evaluated by Scatchard analysis with the computerized program LIGAND[22] (Fig 1, inset). The molecular mass of the vWF subunit (275 kD) was substituted for that of the native, polydisperse, multimerized molecule. The data points fit a straight line, which indicates the existence of a single class of binding sites. The binding was saturable, and a total of 1.60 μg of vWF bound to 10^8 platelets with an apparent dissociation constant (K_d) of 3.43 × 10^{-8} mol/L (9.43 μg/mL). This value is similar, though slightly higher, to that reported for ristocetin-induced binding.[23]

When [^{125}I]vWF (5 μg/mL) was mixed with various amounts of unlabeled vWF and formalin-fixed platelets

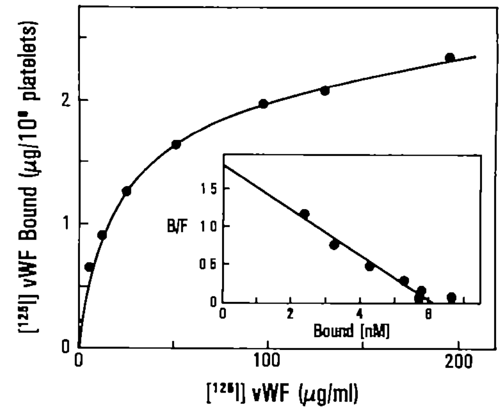

Fig 1. Saturation curve of vWF binding to fixed platelets. Increasing amounts of [^{125}I]vWF (0.28 mCi/mg), as indicated on the abscissa, were mixed with formalin-fixed platelets (10^8/mL); then botrocetin (5 μg/mL) was added and incubated for 30 minutes at room temperature. The amount of [^{125}I]vWF bound to formalin-fixed platelets was then measured as described in Materials and Methods. The values shown in this graph represent total binding. The inset shows a Scatchard-type plot of the experimental data obtained by using the computer-assisted program LIGAND and the points represent specific binding by subtracting the nonspecific binding (calculated as a fitted parameter) from the total binding. The best fit for the experimental points was represented by a straight line, which suggested the existence of a single class of noninteracting binding sites. The relevant binding parameters for this experiment were as folows: [^{125}I]vWF bound at saturation, 1.69 μg/10^8 platelets; K_d, 3.43 × 10^{-8} mol/L (calculated per vWF subunit and assuming a molecular mass of 275 kD); nonsaturable (nonspecific) binding, 0; B/F, bound/free.

(10^8/mL) and binding was initiated by botrocetin, more than 95% of bound radioactivity was displaced by a 50-fold excess of unlabeled ligand (not shown).

The effect of tryptic fragments on botrocetin-induced [^{125}I]vWF binding to platelets. Five tryptic fragments of vWF that had been reduced and *S*-carboxymethylated were evaluated for their effect on botrocetin-induced binding of vWF to platelets. The NH_2-terminal residues of these fragments are as follows[8,9]: the 52/48-kD doublet, Val_{449}; 13 kD, Gln_{290}; 22 kD, Val_{1927}; 41 kD, Thr_{1352} and 55 kD, Asn_{730}. The COOH-terminus of the 52/48-kD fragment corresponded to Lys_{728}.[9] Based on a 275-kD molecular mass for the vWF subunit, these fragments represent ~65% of the entire subunit molecule.

In competitive binding assays, the 52/48-kD fragment completely inhibited [^{125}I]vWF binding to platelets at a concentration of ~2 μmol/L with an IC_{50} of 0.25 μmol/L. The other fragments of vWF did not inhibit at concentrations ~50 μmol/L (Fig 2).

The 52/48-kD fragment also prevented botrocetin-induced platelet aggregation in a dose-dependent manner, with aggregation completely abolished at a concentration of 150 μg/mL (3 μmol/L) (Fig 3). These results indicate that a domain of vWF within the 52/48-kD fragment is necessary for botrocetin-induced platelet agglutination.

The effect of monoclonal antibodies directed against GPIb and GPIIb/IIIa on botrocetin-induced platelet aggregation/agglutination. To determine whether botrocetin-induced agglutination required binding of vWF to GPIb, monoclonal antibodies directed against GPIb were investigated for their ability to block botrocetin-induced platelet agglutination. The two antibodies to GPIb that blocked ristocetin-induced platelet agglutination (AP-1 and LJ-Ib1) also blocked botrocetin-induced agglutination, whereas another anti-GPIb antibody not affecting ristocetin-induced agglutination (LJ-Ib12) had no affect on botrocetin-induced agglutination (Fig 4). Monoclonal antibodies to GPIIb/IIIa that blocked the binding of fibrinogen and vWF (LJ-CP8) or vWF alone (LJ-P5) to this receptor had no effect on botrocetin-induced agglutination.

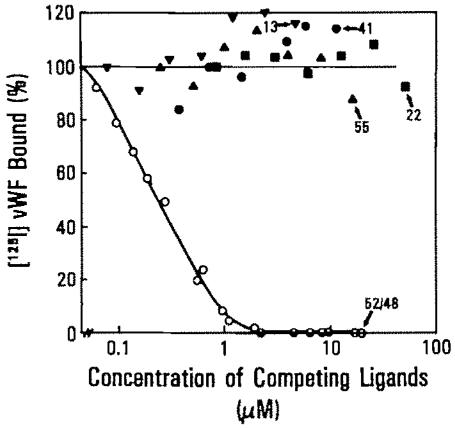

Fig 2. Inhibition of botrocetin-induced vWF binding to fixed platelets by the 52/48-kD fragment. [^{125}I]vWF (5 μg/mL), formalin-fixed platelets (10^8/mL), and various concentrations of reduced and alkylated 52/48-kD (○ ○), 41-kD (● ●), 22-kD (■ ■), 13-kD (▼ ▼) and 55-kD (▲ ▲) tryptic fragments of vWF were incubated for 30 minutes at room temperature after the addition of botrocetin (5 μg/mL), then platelet-bound radioactivity was measured. After subtraction of nonspecific binding, the specific binding was expressed as percentages.

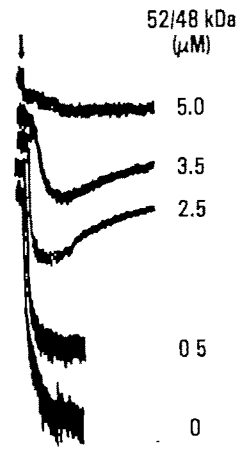

Fig 3. Inhibition of botrocetin-induced platelet aggregation by the 52/48-kD fragment. Formalin-fixed platelets (3 × 10^5/μL), purified vWF (5 μg/mL), and various concentration of a 52/48-kD fragment were mixed in a cuvette for five minutes at 37°C; then botrocetin (5 μg/mL) was added to initiate the platelet agglutination.

DISCUSSION

We have previously demonstrated that the 52/48-kD tryptic fragment of vWF contained the domain that binds to the platelet GPIb receptor. This was true whether binding occurred spontaneously, as is the case for asialo-vWF and vWF from IIB vWD,[24] or binding of normal vWF was induced by ristocetin.[8-11] This 52/48-kD fragment begins with Val_{449} and ends with Lys_{728}[9]; it binds directly to the GPIb molecule without any platelet agonist, and its binding is independent of platelet metabolism.[10] The ability of this fragment to block botrocetin-induced binding to platelets in the experiments reported here shows that the domain of vWF that binds to the GPIb receptor in response to botrocetin falls between residues Val_{449} and Lys_{728}.

Monoclonal antibodies to GPIb that block ristocetin-induced platelet agglutination also blocked botrocetin-induced platelet agglutination. This demonstrated that

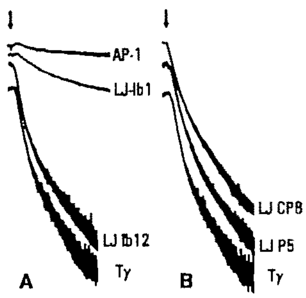

Fig 4. Inhibition of botrocetin-induced platelet agglutination by monoclonal antibodies to GPIb. Purified monoclonal IgGs against GPIb (A) and the GPIIb/IIIa complex (B) were incubated with washed, gel-filtered platelets (3.0 × 10^5/μL) and purified vWF (5 μg/mL) for five minutes at 37°C. Botrocetin was added at the time indicated by arrows. The anti-GPIb monoclonal antibodies AP-1 (50 μg/mL) and LJ-Ib1 (1,000 μg/mL) produced essentially complete inhibition of botrocetin-induced agglutination, whereas LJ-Ib12 (1,000 μg/mL) did not. Neither of the anti-GPIIb/IIIa antibodies LJ-CP8 (200 μg/mL) and LJ-P5 (700 μg/mL) had an effect. Purified antithyroglobulin monoclonal antibody (Tγ) (1,000 μg/mL) served as a control.

botrocetin, like ristocetin, caused binding of vWF to GPIb. However, Howard et al[7] reported that even though people with Bernard-Soulier syndrome are congenitally deficient in GPIb, their platelets were agglutinated by botrocetin. This suggested that an additional platelet binding site for vWF may exist. Our data do not exclude this possibility. However they do make GPIIb/IIIa an unlikely candidate for the secondary site.

Botrocetin induces platelet agglutination in IIA vWD PRP, whereas ristocetin does not. Thus botrocetin cannot be used as a diagnostic reagent for this and perhaps other vWD variants. However, the ability of botrocetin to induce platelet agglutination with IIA vWF, and thus in the absence of large multimers, does not need to be interpreted as showing that botrocetin induces binding through a different vWF domain than does ristocetin. In fact, the reduced and alkylated monomeric 52/48-kD tryptic fragment of vWF binds to the GPIb receptor, which indicates that the domain for binding to GPIb is present on all multimers. However, the larger multimers, as a likely result of their increased valency, have a higher affinity. It is probable that botrocetin simply provides a stronger stimulus for vWF binding than does ristocetin and thus induces agglutination in the absence of large multimers. How these different stimuli induce vWF to interact with the GPIb receptor remains an important unanswered question for our understanding of vWF function.

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Isolation of Lactoferrin cDNA From a Human Myeloid Library and Expression of mRNA During Normal and Leukemic Myelopoiesis

By Thomas A. Rado, Xiping Wei, and Edward J. Benz, Jr

Lactoferrin is a major constituent of polymorphonuclear leukocyte granules and is present in mature neutrophils but not in blasts or promyelocytes. We have isolated a cDNA probe for lactoferrin and used it to study the synthesis of lactoferrin mRNA by normal and leukemic granulocyte precursors. The probe pHL-41 has been subcloned in phage m13 and characterized by restriction endonuclease analysis and nucleic acid sequencing. pHL-41 contains approximately 40% of the coding sequence of the lactoferrin gene. The 3′ untranslated region includes a stop codon and a possible polyadenylation signal. There is a >98% agreement between the cDNA-deduced amino acid sequence and that determined by analysis of the protein. Myeloid cells from normal bone marrow and circulating leukocytes from patients with chronic granulocytic leukemia contain lactoferrin mRNA transcripts that are indistinguishable in size and relative quantity. The human promyelocytic leukemia cell line HL-60 contains no lactoferrin mRNA. Induction of monocytic or granulocytic differentiation fails to induce the synthesis of detectable lactoferrin message. Similarly, studies with the human myeloblastic leukemia cell line PLB-985 reveal the inability of these cells to produce lactoferrin mRNA even under conditions that bring about morphologically demonstrable granulocytic differentiation. These data suggest that granulocytic differentiation in the leukemic cell lines is incomplete or defective. The presence of lactoferrin may play a role in the orderly expression of the genetic program leading to the development of the normal mature granulocyte.

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LACTOFERRIN is an iron-binding protein of approximately 80,000 dalton molecular weight (mol wt) that is expressed in hematopoietic cells in late stages of granulocytic differentiation and is also found in human exocrine secretions.[1-4] Several lines of evidence suggest that this protein is involved in the downregulation of myelopoiesis[5,6] through an effect mediated by responsive mononuclear cells.[7] The in vivo function of lactoferrin as a regulatory molecule is still controversial.[8-10] Serum levels of the protein have recently been proposed as a marker of bone marrow engraftment after transplantation[11,12] and a marker of granulocyte activation.[13,14]

The acute nonlymphocytic leukemias are characterized by the replacement of normal myelopoietic tissue by cells displaying defective maturation. These cells have morphological and cytochemical characteristics typical of early granulocytic precursors. Myeloblasts predominate in acute myeloblastic leukemia (AML) and promyelocytes in the acute promyelocytic form (APL). Metabolic studies on AML and APL cells indicate that they synthesize no lactoferrin.[15] Staining of bone marrow with fluorescence-labeled monoclonal antibody to lactoferrin demonstrates that normal myeloblasts and promyelocytes also have no immunoreactive material although cells of later stages of granulocytic maturation stain positively for the protein (L. Heck, personal communication). Similarly, in chronic granulocytic leukemia (CGL), a disease characterized by expansion of the granulocyte population and their maturing precursors in peripheral blood, only recognizably mature cells (ie, metamyelocytes, bands, and neutrophils) contain lactoferrin. The exact stage at which synthesis begins, however, is unknown. These data strongly suggest that lactoferrin is a gene whose expression during myelopoiesis is developmentally modulated.

Study of the regulation of lactoferrin transcription and its role in the regulation of myeloid maturation has been impeded by the absence of a cloned probe for the gene. We report here the molecular cloning of lactoferrin cDNA from human myeloid cells and the use of the probe to assess the expression of lactoferrin mRNA by normal and leukemic myeloid precursors. Transcription of lactoferrin by the human leukemia cell lines HL-60 and PLB-985, both of which are capable of limited granulocytic maturation in the presence of inducing agents,[16,17] has also been investigated.

MATERIALS AND METHODS

Cell lines and granulocytes. The HL-60 cell line, originally isolated from a patient with APL[18] was a kind gift of Dr Hal Broxmeyer PLB-985 is a new human myeloid leukemia cell line that is characterized by a less mature phenotype than HL-60 and the ability to undergo granulocytic differentiation in the presence of chemical inducing agents.[17] Peripheral granulocytes from leukemia patients were obtained from routine diagnostic specimens. Granulocytes from CGL peripheral blood were prepared from leukapheresis specimens obtained with the patients' informed consent.

HL-60 and PLB-985 cells were propagated in RPMI 1640 medium containing 5% Nu-Serum (Collaborative Bioresearch, Bethesda, MD). Induction of granulocytic maturation was accomplished with dimethyl sulfoxide (DMSO) or dibutyryl cyclic adenosine monophosphate (Bt_2 cAMP) as previously described.[19,20] Monocytic maturation of the cells was induced with phorbol myristate acetate (PMA).[19] Induction of differentiation was routinely assessed by morphological examination of the cells under Wright's stain, determination of the percentage of adherent cells in the population, and demonstration of decreased transcription of the *myc* oncogene[21,22] as determined by Northern blot analysis.

RNA extraction. White cells from human blood and leukapheresis specimens were purified from buffy coat by a single wash with

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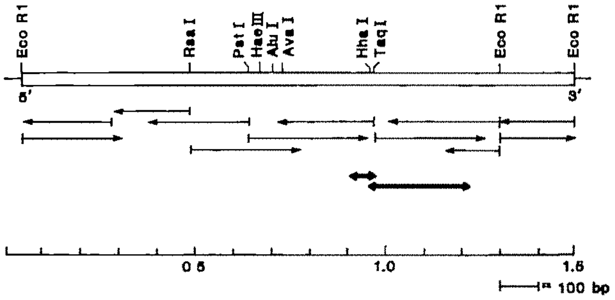

Fig 1. Sequencing strategy for clone pHL-41. Significant enzymes are indicated above the map. Restriction fragments were purified on polyacrylamide gels and subcloned into M13mp18 and M13mp19. Arrow heads indicate the direction of sequencing. Each fragment was sequenced in at least two independent reactions. →, Dideoxy sequence; ↔, bidirectionally sequenced (Maxam-Gilbert).

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   1  AAATCCCAACAAAGCAGTGACCCTGATCCTAACTGTGTGGATAGACCTGTGGAAGGATATCTTGCTGTGGCGGTGGTTAGG   81
      ---------+---------+---------+---------+---------+---------+---------+---------+-
 428  LysSerGlnGlnSerSerAspProAspProAsnCysValAspArgProValGluGlyTyrLeuAlaValAlaValValArg  454

  82  AGATCAGACACTAGCCTTACCTGGAACTCTGTGAAAGGCAAGAAGTCCTGCCACACCGCCGTGGACAGGACTGCAGCGTGG  162
      --------+---------+---------+---------+---------+---------+---------+---------+--
 455  ArgSerAspThrSerLeuThrTrpAsnSerValLysGlyLysLysSerCysHisThrAlaValAspArgThrAlaAlaTrp  481
                                                                                 Gly

 163  AATATCCCCATGGGCCTGCTCTTCAACCAGACGGGCTCCTGCAAATTTGATGAATATTTCAGTCAAAGCTGTGCCCCTGGG  243
      -------+---------+---------+---------+---------+---------+---------+---------+---
 482  AsnIleProMetGlyLeuLeuPheAsnGlnThrGlySerCysLysPheAspGluTyrPheSerGlnSerCysAlaProGly  508

 244  TCTGACCCGAGATCTAATCTCTGTGCTCTGTGTATTGGCGACGAGCAGGGTGAGAATAAGTGCGTGCCCAACAGCAATGAG  324
      ------+---------+---------+---------+---------+---------+---------+---------+----
 509  SerAspProArgSerAsnLeuCysAlaLeuCysIleGlyAspGluGlnGlyGluAsnLysCysValProAsnSerAsnGlu  535
                                                   Glu

 325  AGATACTACGGCTACACTGGGGCTTTCCGGTGCCTGGCTGAGAATGCTGGAGACGTTGCATTTGTGAAAGATGTCACTGTC  405
      -----+---------+---------+---------+---------+---------+---------+---------+-----
 536  ArgTyrTyrGlyTyrThrGlyAlaPheArgCysLeuAlaGluAsnAlaGlyAspValAlaPheValLysAspValThrVal  562

 406  TTGCAGAACACTGATGGAAATAACAATGAGGCATGGGCTAAGGATTTGAAGCTGGCAGACTTTGCGCTGCTGTGCCTCGAT  486
      ----+---------+---------+---------+---------+---------+---------+---------+------
 563  LeuGlnAsnThrAspGlyAsnAsnAsnGluAlaTrpAlaLysAspLeuLysLeuAlaAspPheAlaLeuLeuCysLeuAsp  589

 487  GGCAAACGGAAGCCTGTGACTGAGGCTAGAAGCTGCCATCTTGCCATGGCCCCGAATCATGCCGTGGTGTCTCGGATGGAT  567
      ---+---------+---------+---------+---------+---------+---------+---------+-------
 590  GlyLysArgLysProValThrGluAlaArgSerCysHisLeuAlaMetAlaProAsnHisAlaValValSerArgMetAsp  616

 568  AAGGTGGAACGCCTGAAACAGGTGCTGCTCCACCAACAGGCTAAATTTGGGAGAAATGGATCTGACTGCCCGGACAAGTTT  648
      --+---------+---------+---------+---------+---------+---------+---------+--------
 617  LysValGluArgLeuLysGlnValLeuLeuHisGlnGlnAlaLysPheGlyArgAsnGlySerAspCysProAspLysPhe  643

 649  TGCTTATTCCAGTCTGAAACCAAAAACCTTCTGTTCAATGACAACACTGAGTGTCTGGCCAGACTCCATGGCAAAACAACA  729
      -+---------+---------+---------+---------+---------+---------+---------+---------
 644  CysLeuPheGlnSerGluThrLysAsnLeuLeuPheAsnAspAsnThrGluCysLeuAlaArgLeuHisGlyLysThrThr  670

 730  TATGAAAAATATTTGGGACCACAGTATGTCGCAGGCATTACTAATCTGAAAAAGTGCTCAACCTCCCCCCTCCTGGAAGCC  810
      +---------+---------+---------+---------+---------+---------+---------+---------+
 671  TyrGluLysTyrLeuGlyProGlnTyrValAlaGlyIleThrAsnLeuLysLysCysSerThrSerProLeuLeuGluAla  697
                                                      Arg

 811  TGTGAATTCCTCAGGAAGTAAAACCGAAGAAGATGGCCCAGCTCCCCAAGAAAGCCTCAGCCATTCACTGCCCCCAGCTCT  891
      ---------+---------+---------+---------+---------+---------+---------+---------+-
 698  CysGluPheLeuArgLysEnd
                     703

 892  TCTCCCCAGGTGTGTTGGGGCCTTGGCTCCCCTGCTGAAGGTGGGGATTGCCCATCCATCTGCTTACAATTCCCTGCTGTC  972
      --------+---------+---------+---------+---------+---------+---------+---------+--

 973  GTCTTAGCAAGAAGTAAAATGAGAAATTTTGTTGAAAAAAAAAAAAAAAAA  1023
      -------+---------+---------+---------+---------+---
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Fig 2. Nucleotide sequence of clone pHL41 and deduced amino acid sequence. Nucleotides are numbered from the 5' end of the clone. Amino acid residues are numbered according to the sequence of Metz-Boutigue et al.[29] Discrepancies between the deduced and published amino acid sequence are indicated by squares, with the published residue (or its absence) given below. A possible polyadenylation signal begins at nucleotide 984.

phosphate-buffered saline (PBS). Erythrocytes were removed by treatment of the cell pellet with 10 vol of 0.2% NaCl for 30 seconds at 0°C followed by the addition of an equal volume of 1.6% NaCl. Centrifugation at 1,000 g for ten minutes provided a pellet that was composed entirely of nucleated cells. Cultured cells in suspension were washed once with ice cold PBS and pelleted as before. Adherent cells (eg, HL-60 and PLB-985 grown in the presence of PMA) were lysed directly in their culture flasks.

Total cellular RNA was extracted from cultured cells and human neutrophils by rapid lysis of frozen material in guanidinium isothiocyanate[23] and was further purified by pelleting through 5.7 mol/L cesium chloride.[24] Polyadenylated RNA was prepared by two cycles of chromatography over oligo-dT-cellulose (Pharmacia Fine Chemicals, Piscataway, NJ).[25]

cDNA synthesis and library screening. Double-stranded cDNA was synthesized by the method of Gubler and Hoffman[26] by using as a template 10 μg of poly-A RNA from human CGL granulocytes. *Eco*RI linkers were added, and the cDNA was ligated into λgt10 and packaged in vitro by using commercial packaging extract (Stratagene, San Diego, CA) and the instructions of the manufacturer. The library prepared in this manner contained $>10^6$ independent clones. The unamplified library was plated at high density on *Escherichia coli* C600 hflA$^-$. Plaques were transferred to nylon membranes (GeneScreen Plus, NEN-DuPont, Boston) coated with bacteria for in situ signal amplification.[27] The membranes were hybridized to an oligonucleotide probe, with sequence 5′-D(GAYTGTCCN-GAYAARTT)-3′ corresponding to the amino acid sequence of residues 638 to 643 of human colostrum lactoferrin.[28] The filters were washed at high stringency under conditions that make DNA strand dissociation (T_d) independent of guanine plus cytosine (GC) content.[29] Sixteen independent clones were isolated and subcloned into M13mp18 and M13mp19[30] for nucleotide sequencing.

DNA sequencing and analysis. Cloned inserts were sequenced in multiple directions by using the dideoxy chain termination method of Sanger with Klenow fragments of DNA polymerase I[31] and avian myeloblastosis virus (AMV) reverse transcriptase.[32] The sequencing reactions were modified for the use of ^{35}S-labeled nucleotides and buffer gradient gels.[33] In addition, some regions were sequenced bidirectionally by using the chemical method of Maxam and Gilbert.[34] The sequencing strategy is shown in Fig 1. Sequence data were analyzed by using the UWCG computer program.[35]

Hybridization analysis of RNA. RNA was analyzed by Northern blotting from formamide/formaldehyde gels[36] and hybridized to ^{32}P-labeled DNA probes. The probes included the internal *Eco*RI-*Pst*I fragment of pHL-41 (Fig 1), a cDNA probe for γ-actin kindly provided by L. Kedes,[37] and a cDNA probe for *c-myc*.[38] The probes were labeled by the random oligonucleotide technique.[39]

RESULTS

Isolation and sequence of pHL-41. Sequence analysis (Fig 2) of the lactoferrin inserts obtained on this screening of the library revealed that one clone, pHL-41, contained 1,023 bases, including the coding sequence for amino acids 428 to 703, a stop codon, a possible polyadenylation signal, and a poly-A tail. The amino acid sequence deduced from the cDNA differed in three residues from that reported by protein sequence determination.[28]

Expression of lactoferrin mRNA by myeloid cells. The ability of myeloid cells to express lactoferrin mRNA transcripts was determined by Northern blot analysis of polyadenylated RNA from various sources. Normal human bone marrow granulocytes and granulocytes from CGL peripheral blood synthesized transcripts of the same mol wt. The intensity of hybridization of the lactoferrin band and a concurrently hybridized γ-actin band has been found to be comparable in CGL and in normal bone marrow–derived granulocyte precursors (Fig 3, lanes 1 and 2).

Interestingly, cells from the same CGL patient that had been obtained after blastic transformation (myeloid type) had occurred displayed no hybridization to the lactoferrin probe (Fig 3, lane 3). In addition, AML and APL blasts also synthesized no lactoferrin mRNA (data not shown).

The HL-60 and PLB-985 cell lines were studied because of their capacity to undergo maturation in a granulocytic or monocytic direction under chemical stimulation. We have previously shown that lactoferrin translation product is undetectable in DMSO- or *cis*-retinoic acid–induced HL-60 cells.[15] These studies have now been extended to include a determination of the ability of these cells to express lactoferrin transcripts. Poly-A RNA from uninduced and induced cells was hybridized to a lactoferrin probe as described earlier. In no instance was normal- or aberrant-sized transcript detected. In all observed instances, synthesis of actin mRNA was constitutive as predicted[37] (Fig 3, lanes 4 to 8). The γ-actin hybridization acted as control to rule out the possibility that the absence of lactoferrin signal (lanes 4 to 8) was due to technical factors affecting the efficiency of mRNA transfer; it also acted as a standard against which the intensity of the lactoferrin band (lanes 1 and 2) could be compared.

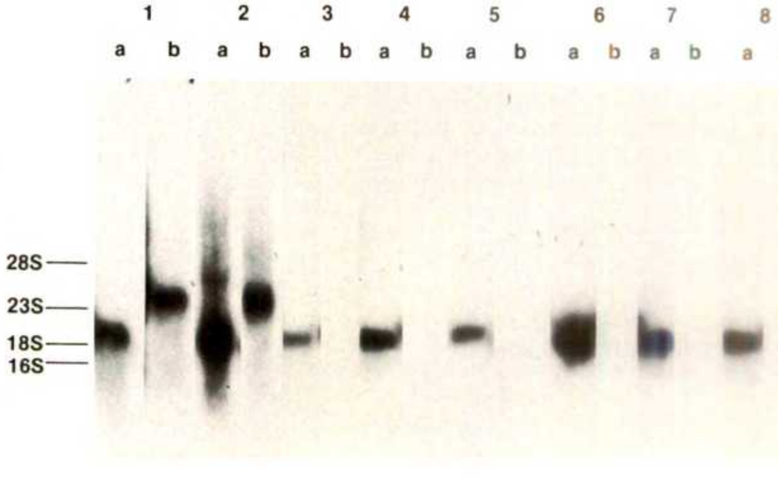

Fig 3. Northern blot analysis of lactoferrin gene expression. Each lane contains 4 μg of poly-A RNA. Lanes a were hybridized to the 2.1 kilobase (kb) *Bam*HI insert of pHFγA-1.[31] Lanes b were probed with the 0.8 kb *Pst-Eco* fragment of pHL-41. Sources of RNA: 1, normal bone marrow; 2, CGL peripheral blood leukocytes; 3, CGL blast crisis peripheral blood leukocytes; 4, HL-60 uninduced; 5, HL-60 induced for 48 hours with PMA; 6, HL-60 induced for three days with Bt_2 cAMP; 7, PLB-985 induced for 48 hours with PMA; 8, PLB-985 induced for three days with Bt_2 cAMP. Induction conditions and RNA extraction are as described in Materials and Methods. Mol wt markers are indicated at the left.

DISCUSSION

We report here the successful cDNA cloning of human neutrophil lactoferrin. The clone has been characterized by nucleotide sequence analysis and encompasses approximately 40% of the coding region as well as the 3′ terminus. The clone was used as a probe to study the expression of the gene in normal and leukemic cells and in two leukemia cell lines capable of limited granulocytic differentiation. The results reported here indicate that myeloid cell populations containing precursors more mature than promyelocytes synthesize lactoferrin mRNA. Thus, normal bone marrow is indistinguishable from CGL peripheral blood with respect to the abundance and size of the major lactoferrin mRNA transcript. In concurrence with the absence of morphologically recognizable differentiation, the lactoferrin transcript was not found in the peripheral white cells of CGL blast crisis or in AML and APL cells.

The absence of lactoferrin transcripts in undifferentiated and chemically induced (differentiated) HL-60 and PLB-985 cells indicates that in vitro maturation of these cells is defective. These lines clearly display some characteristics of maturation such as morphological increases in nuclear pyknosis, expression of the fMet-Leu-Phe receptor,[40] and decreased *myc* transcription.[21,22] Their inability to express a developmentally modulated gene that characteristically begins expression at the myelocyte stage with progressive increases to the fully mature myeloid elements suggests substantial arrest of at least portions of the orderly sequence of genetic modulation integral to cell maturation.

Delineation of the mechanisms responsible for this persistent defect should provide insights into the process of maturation arrest that typifies malignant transformation. The successful cloning of this cDNA probe will provide the basis for exploring this gene and its control mechanisms.

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Receptor-Mediated Monocytoid Differentiation of Human Promyelocytic Cells by Tumor Necrosis Factor: Synergistic Actions With Interferon-γ and 1,25-Dihydroxyvitamin D_3

By J. Brice Weinberg and James W. Larrick

Human myeloid leukemia cells respond to various signals by differentiating to more mature cells. This study was designed to evaluate the effects of a mononuclear phagocyte-derived factor, tumor necrosis factor/cachectin (TNF), on the proliferation and differentiation of the human cell lines HL-60 (promyelocytic) and U937 (monoblastic), and to characterize TNF receptors on these cells. TNF had no effect on HL-60 cell growth or thymidine incorporation, but it markedly inhibited that of U937 cells. HL-60 cells treated with TNF formed osteoclast-like polykaryons and developed nonspecific esterase positivity. In a dose-dependent fashion, TNF enhanced HL-60 cell nonspecific esterase activity, H_2O_2 production, NBT reduction, and acid phosphatase content. Together, TNF and interferon-γ (IFN-γ) additively and synergistically caused increases in these activities as well as the expression of HLA-DR and the monocyte antigens LeuM3 (CDw14) and OKM1 (CD11). TNF also synergistically enhanced the differentiating effects of 1,25-dihydroxyvitamin D_3. The potentiating actions of D_3 of IFN-γ on the TNF effect were maximal when the two agents were present together throughout the incubation, and pretreatment with TNF augmented the subsequent response to D_3, but not IFN-γ. HL-60 and U937 cells bound ^{125}I-labeled TNF specifically, rapidly, and reversibly with binding constants of 227 and 333 pmol/L and receptors per cell of 4,435 and 6,806 for HL-60 and U937, respectively. Scatchard plots were linear, which suggested single classes of receptors. HL-60 TNF receptors were not changed by a three-day treatment with IFN-γ or D_3. U937 and HL-60 cells internalized and degraded ^{125}I-labeled TNF to comparable degrees. TNF has differing effects on HL-60 and U937 cells that are apparently mediated through comparable high-affinity TNF receptors. The unique responses of different cell types to TNF may be due to postreceptor factors.

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HUMAN MYELOID LEUKEMIA cells have been noted to differentiate in vitro in response to several different agents.[1,2] Compounds such as dimethylsulfoxide, retinoic acid, cyclic adenosine monophosphate (AMP), and cytosine arabinoside cause myeloid differentiation,[1-4] whereas others including 1α,25-dihydroxyvitamin D_3 (D_3), phorbol diesters, interferon-γ (IFN-γ), and supernatants from mitogen-stimulated mononuclear cells cause differentiation to monocyte-like cells.[1,2,5-9] It was recently demonstrated that the monokine tumor necrosis factor/cachectin (TNF) also induced differentiation of myeloid leukemia cells to monocyte-like cells.[10,11] TNF is a protein secreted by mononuclear phagocytes in response to a variety of stimuli, in particular endotoxin (lipopolysaccharide, LPS).[12] It was initially found by Carswell et al in the serum of mice infected with bacillus Calmette-Guerin treated with intravenous LPS.[13] The macrophage-derived protein is static or lytic for most (but not all) tumor cell line cells and has a relative sparing for nontransformed cells.[12,14,15] In addition to its ability to influence tumor cell growth, TNF also modulates the function of normal cells including adipocytes,[14,16] polymorphonuclear leukocytes,[17-19] endothelial cells[20-22] fibroblasts,[23] and mononuclear phagocytes.[24-26] Several cell types have been noted to have specific membrane receptors for TNF.[14,16,19,27,28] These receptors appear to be necessary for but not sufficient to ensure that a particular cell will respond in a measurable way to the TNF. The purpose of this work was to examine the effects of TNF on human leukemia cell growth and differentiation in vitro and to characterize leukemia cell receptors for TNF. Results show that the TNF causes a dose-dependent differentiation of promyelocytic HL-60 cells to monocytoid cells without affecting their viability or proliferative rate and that the TNF acts in synergy with IFN-γ and D_3 to produce these effects. Also both HL-60 and U937 cells have high-affinity membrane receptors for TNF.

MATERIALS AND METHODS

Medium and serum. All cultures were done in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with dextrose (1 mg/mL), 20 mmol/L HEPES buffer, penicillin (100 U/mL), and streptomycin (100 μg/mL).[5,29] Fetal bovine serum (FBS) was from Sterile Systems (Logan, UT). By limulus amebocyte lysate testing, the materials contained <200 pg/mL of LPS.

Culture methods. The HL-60 and U937 cell lines were from the American Tissue Culture Collection (Rockford, MD). They were used during passages 30 to 68. Maintenance cultures were passed two to three times each week after initial seedings of 2.5 to 5.0 × 10^5/mL in 10% FBS. In experiments, the cells were cultured at 1 to 2 × 10^5/mL with the various additives at 37°C with 5% CO_2 and 100% humidity in 25- or 75-cm^2 surface area plastic culture flasks (Becton Dickinson, Oxnard, CA) for four to seven days. Cell viability was determined by trypan blue exclusion.

Hydrogen peroxide and nitroblue tetrazolium assays. The production of hydrogen peroxide (H_2O_2) in the presence or absence of 200 nmol/L phorbol myristate acetate (PMA) was determined by using horseradish peroxidase and phenol red and a microtiter plate reader as described previously.[5,30] Nitroblue tetrazolium (NBT) reduction by the cells in response to 200 nmol/L PMA was assessed by visually counting safranin-counterstained slides.[4,31]

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Morphology and cytochemistry. After appropriate cultures, the cells were collected onto glass slides by using a Shandon cytocentrifuge (Shandon Southern, Astmoor, England). Wright's, nonspecific esterase (α-napthylbutyrate esterase), and acid phosphatase (with and without 27 mmol/L sodium tartrate) stains were done as described before.[32]

Cell surface antigen studies. After cultures with the additives indicated, the cells were processed for indirect immunofluorescent staining and analysis on an EPICS V fluorescent cell analyzer (Coulter) as described before.[7] Antibodies against HLA-DR, LeuM3 (CDw14), and LeuM2 were from Becton Dickinson (Mountain View, CA), and OKM1 (CD11) was from Ortho Diagnostics (Raritan, NJ).

Acid phosphatase and lysozyme assays. After cells were dissolved in 0.05% Triton X-100, acid phosphatase was measured by using *p*-nitrophenol phosphate as a substrate, and protein was measured as described before.[32] Lysozyme activity was measured by using *Micrococcus lysodekticus* as a substrate with egg white lysozyme as a standard.[33]

Thymidine incorporation assays. U937 and HL-60 cells were cultured at 0.8 × 10^4 and 1.0 × 10^4/microtiter plate chamber, respectively, in 0.2 mL RPMI 1640 with 10% FBS with different additives for 60 hours. Then, 1 μCi of tritiated thymidine (^{3}HTdR, 2 Ci/mmol) was added to each chamber, and after four further hours of culture, the cells were harvested by using a multiple automated sample harvester, deposited onto glass fiber filter paper, and washed with distilled water.[5] The filter disks were then counted in liquid scintillant.

Receptor binding assay. TNF was labeled to a specific activity of approximately 0.9 μCi/μg protein by using 1,3,4,6-tetrachloro-3α-6α-diphenylglycoluril (Iodo-gen; Pierce Chemical Co, Rockford, IL) and ^{125}I. The labeled TNF had full bioactivity as judged by its ability to inhibit U937 cell proliferation and to lyse L929 cells in the presence of 1 μg/mL actinomycin D. Three × 10^6 of the cells were incubated with various concentrations of labeled TNF in a final volume of 0.2 mL RPMI 1640 with 10% FBS. To determine nonspecific binding of the labeled TNF, experiments were done in

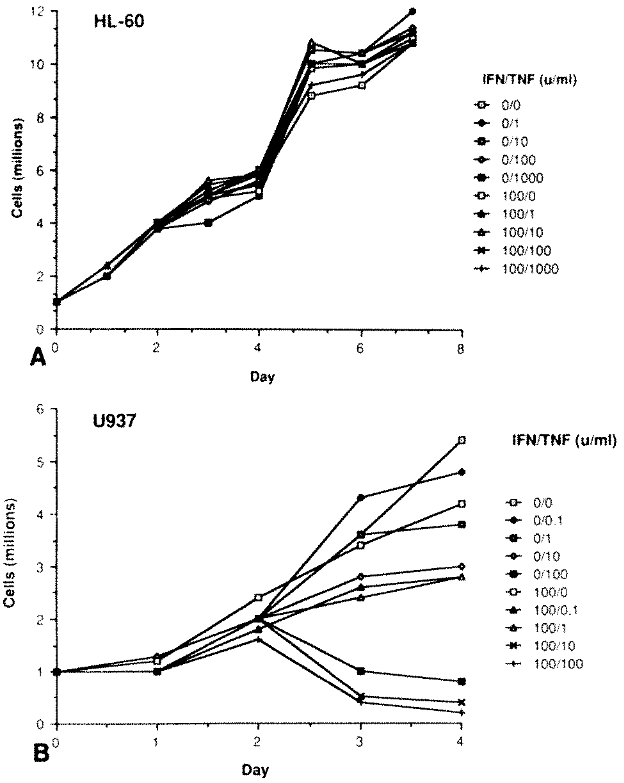

Fig 1. HL-60 and U937 cell counts during incubation with IFN-γ and/or TNF. The counts are means of triplicate samples from an experiment representative of four comparable experiments done with HL-60 and two with U937.

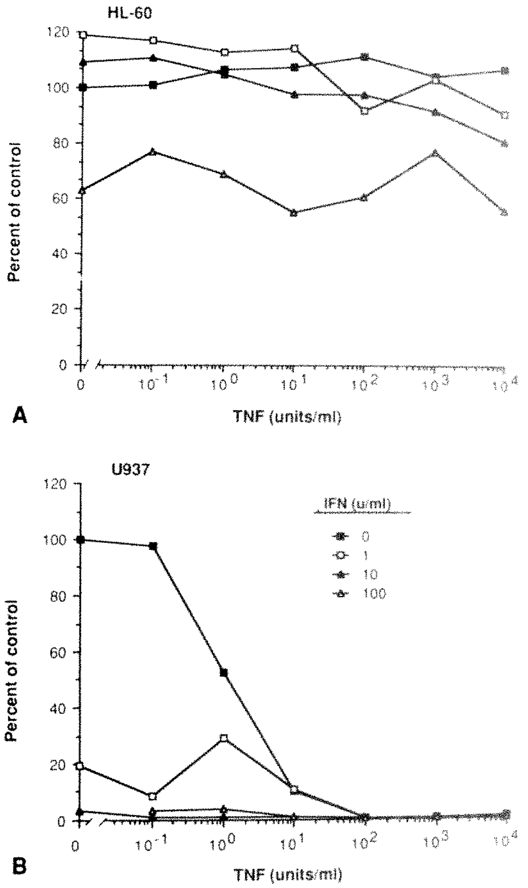

Fig 2. HL-60 and U937 ^{3}H-TdR incorporation after four days of culture with IFN-γ and/or TNF. The numbers are means of triplicate samples from an experiment representative of four comparable experiments done.

Table 1. Induction of Multinuclearity in HL-60 Cells by TNF and IFN-γ

TNF*	IFN-γ*	Multinucleated (%)	Nuclei/Polykaryon	Multinuclearity Index†
0	0	0.0 ± 0.0‡	0.0 ± 0.0	0.0 ± 0.0
10	0	4.2 ± 1.5	2.7 ± 0.3	10.2 ± 3.2
100	0	3.7 ± 0.3	2.5 ± 0.2	8.8 ± 0.2
1,000	0	6.2 ± 0.5	2.2 ± 0.2	12.7 ± 1.2
0	100	2.4 ± 1.8	2.4 ± 0.2	5.4 ± 4.1
10	100	7.7 ± 3.0	3.3 ± 0.3	20.4 ± 6.9
100	100	10.2 ± 1.5	3.6 ± 0.2	29.1 ± 3.0
1,000	100	18.5 ± 4.1	3.4 ± 0.2	42.4 ± 7.4

*Units per milliliter of TNF or IFN-γ present through the five-day culture period.

†Percentage of all nuclei that occur within multinucleated cells.

‡Mean ± SEM of triplicate samples from one of five comparable experiments done.

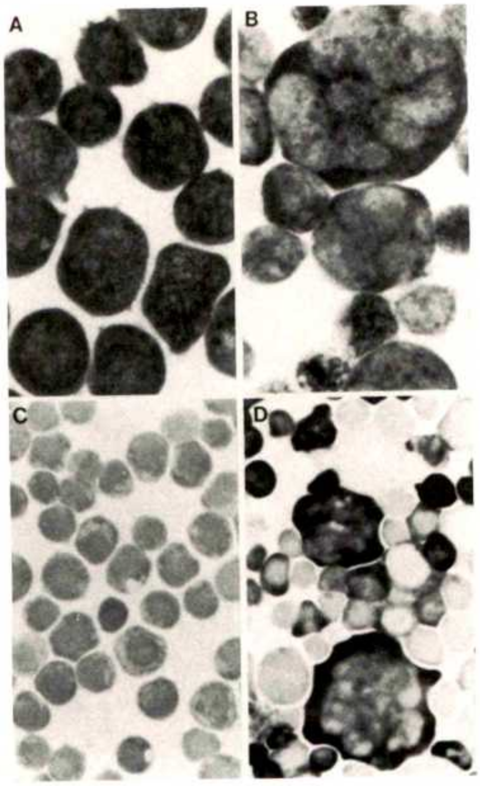

Fig 3. Photomicrographs of HL-60 cells cultured with 0 (A and C) or 1,000 U/mL (B and D) TNF for four days. A and B are stained with Wright's stain (original magnification × 600; current magnification × 396) and C and D with nonspecific esterase stain (original magnification × 300; current mangification × 198). Cells staining positive for nonspecific esterase are gray-black.

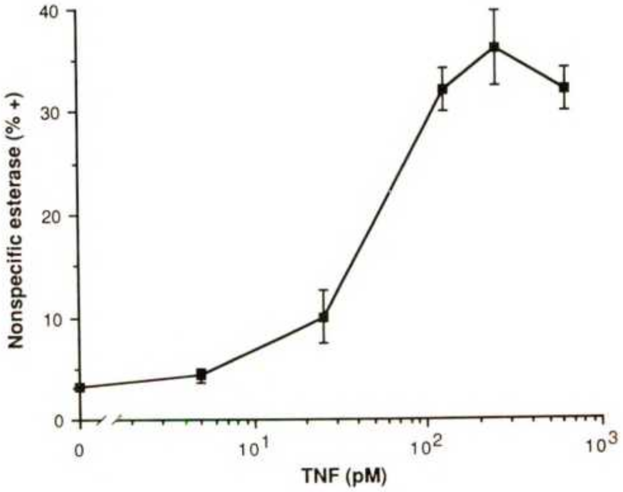

Fig 4. Nonspecific esterase positivity in HL-60 cells cultured four days with varying doses of TNF. The points show means ± SEM from triplicate samples of an experiment representative of 12 comparable experiments done. Note that 100 U/mL of TNF is 580 pmol/L.

the absence and presence of 500 nmol/L unlabeled TNF. All results are presented as specific binding (the difference between ^{125}I-labeled TNF bound in the absence and presence of the excess, unlabeled TNF). Nonspecific binding was generally linear with respect to the concentration of ^{125}I-labeled TNF added and at maximum was <30% of ^{125}I-labeled TNF bound or 2% of ^{125}I-labeled TNF added. The assays were done in triplicate, and all cells and additions were allowed to reach the desired experimental temperature before addition to the tubes. Scatchard analysis was done as described before.[30] To determine the percentage of ^{125}I-labeled TNF that had been

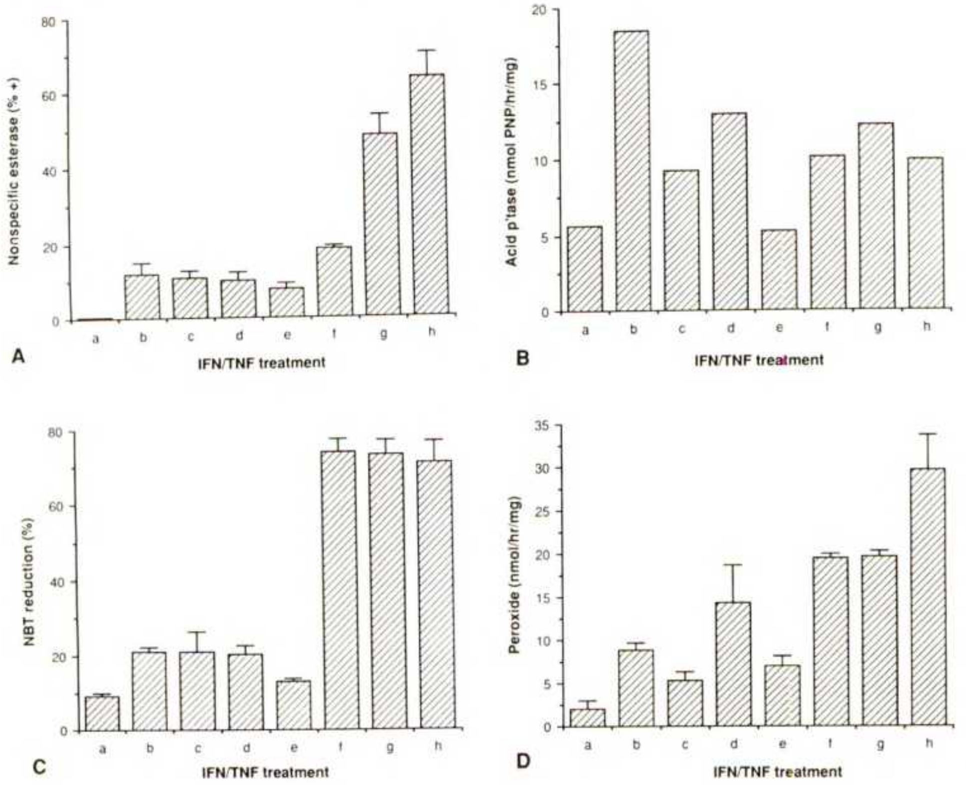

Fig 5. Nonspecific esterase positivity (A), acid phosphatase content (B), PMA-stimulated NBT reduction (C), and PMA-stimulated H_2O_2 production (D) by HL-60 cells after four days of culture with IFN-γ and/or TNF. The bars show means ± 1 SEM of triplicate samples of experiments representative of several comparable experiments done (14 in A, three in B, five in C, and 14 in D).

Table 2. Cell Surface Antigen Analysis of HL-60 Cells Treated With TNF With or Without IFN-γ or D_3

Treatment*			Percent Positive†					Mean Channel Fluorescence‡				
TNF	IFN-γ	D3	P3	HLA-DR	OKM1	LeuM2	LeuM3	P3	HLA-DR	OKM1	LeuM2	LeuM3
0	0	0	2	32	6	2	8	5	14	8	6	13
10	0	0	7	43	14	9	21	7	15	14	9	30
100	0	0	8	86	24	8	49	6	52	25	7	64
0	100	0	9	50	18	12	25	16	49	11	9	28
10	100	0	13	87	22	22	79	21	102	45	12	157
100	100	0	9	76	61	18	85	18	67	50	9	173
0	0	5	5	42	15	9	29	6	20	16	9	40
10	0	5	8	—	24	13	40	7	—	23	13	53
100	0	5	11	66	55	25	79	7	36	52	19	136

*TNF (U/mL), IFN-γ (U/mL), and/or D_3 (nmol/L) present through the four-day culture.

†Percentage of cells with fluorescence higher than the cutoff based on P3 control.

‡Mean channel fluorescence of the positive cells (arbitrary units from logarithmic scale of fluorescence intensity).

internalized, 1,000 pmol/L ^{125}I-labeled TNF was bound to the cells for two hours at 0°C. The cells were then washed twice with 0°C RPMI–10% FBS and incubated for varying periods of time at 0°C or 37°C. Then washed cells were treated for five minutes at 0°C with 0.05 mol/L glycine HCl/0.15 N NaCl buffer (pH 3) to elute the noninternalized ligand. Supernatants and cell pellets were counted for ^{125}I. Supernatants were also analyzed for degraded ^{125}I-labeled TNF by determining the proportion of ^{125}I precipitable by 10% trichloroacetic acid.

Materials. ^{3}HTdR and ^{125}I-sodium were from New England Nuclear (Boston), purified, recombinant human IFN-γ[34] from Genentech (S San Francisco, CA); and purified, recombinant human TNF[35,36] from Cetus Corp (Emeryville, CA). All other reagents were from Sigma Chemical Co (St Louis). As determined by limulus amebocyte lysate assays, the IFN-γ contained <10 ng/mg protein and the TNF, <112 ng/mg protein, which signified final concentrations of LPS <112 pg/mL in assays using the highest doses of IFN-γ and TNF.

RESULTS

Cell proliferation: HL-60 proliferation was not affected by the TNF and/or IFN-γ (Fig 1A). When assessed for their abilities to incorporate ^{3}HTdR on day 3, the HL-60 cells were affected none or little by TNF and only slightly by IFN-γ (Fig 2A). By day 3, TNF and/or IFN-γ slowed U937 proliferation as assessed by the rates of increase in cell number (Fig 1B) and the ^{3}HTdR incorporation (Fig 2B). A concentration of approximately 1 U/mL of TNF (~6 pmol/L) inhibited U937 ^{3}HTdR incorporation by 50%.

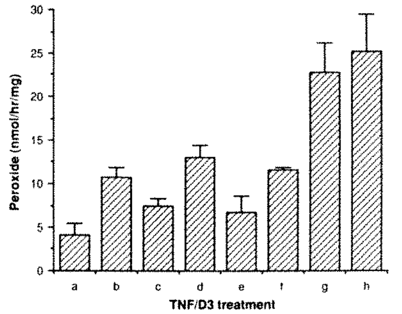

Fig 6. PMA-stimulated H_2O_2 production by HL-60 cells after four days of culture with D_3 and/or TNF. The bars show means ± 1 SEM of triplicate samples from an experiment representative of four comparable experiments done.

Cell morphology and cytochemistry. As we have reported before, IFN-γ induced multinuclearity in HL-60 and U937 cells.[7] The TNF also induced polykaryon formation and appeared to work synergistically with IFN-γ in causing this phenomenom (Table 1 and Fig 3). The nuclear chromatin of the cells was slightly more coarse in the treated cells, but nucleoli were still visible in most cells. The cells did not become adherent after the treatments. In a dose-dependent fashion, TNF enhanced HL-60 expression of the enzyme nonspecific esterase (Fig 4), and TNF and IFN-γ were synergistic in their ability to cause this increase (Fig 5A). All U937 cells were natively positive for nonspecific esterase, but TNF and IFN-γ caused a qualitative increase in activity (data not shown). In the HL-60 cells, most of the polykaryons were positive for nonspecific esterase, but some were negative. The treatments with TNF and IFN-γ caused a qualitative increase in the expression of acid phosphatase in HL-60 and U937 cells. This was apparent in mononuclear as well as multinucleated cells. The acid phosphatase was totally inhibited by tartrate in the mononuclear and multinucleated cells. Acid phosphatase measured in cell lysates demonstrated the increases more quantitatively (Fig 5B). No

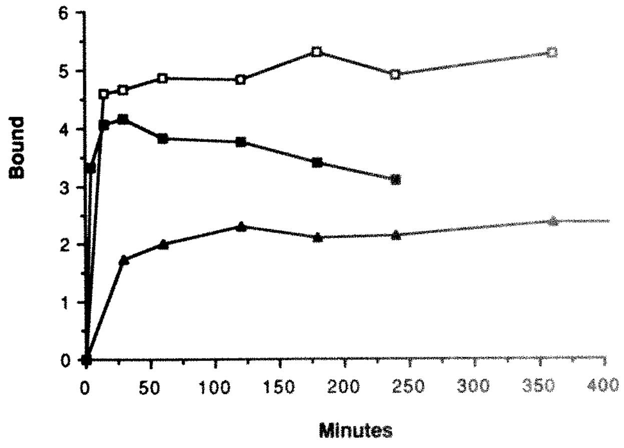

Fig 7. Time course for binding of 500 pmol/L ^{125}I-labeled TNF to HL-60 and U937 cells at different temperatures. The values represent the means (fmol/10^6 cells) of triplicate samples. The value for the 1,080-minute samjple at 4°C (not shown) was 2.3. This is representative of two comparable experiments done.

Table 3. Influence of Sequence of Addition of TNF, D_3, and IFN-γ on HL-60 Cell Differentiation

TNF and IFN-γ			TNF and D_3		
Days 0-3	Days 4-6	H_2O_2 Production*	Days 0-3	Days 4-6	H_2O_2 Production*
0	0	3.8 ± 1.3	0	0	5.0 ± 1.2
TNF	0	7.8 ± 2.7	TNF	0	13.9 ± 2.5
0	TNF	9.5 ± 0.9	0	TNF	16.7 ± 2.5
IFN-γ	0	11.3 ± 0.8	D_3	0	5.9 ± 3.2
0	IFN-γ	13.4 ± 0.7	0	D_3	12.3 ± 2.4
TNF	IFN-γ	15.6 ± 5.4	TNF	D_3	37.1 ± 5.4
IFN-γ	TNF	11.3 ± 0.7	D_3	TNF	4.3 ± 1.4
TNF/IFN-γ	0	8.9 ± 0.4	TNF/D_3	0	14.8 ± 2.0
0	TNF/IFN-γ	13.9 ± 1.2	0	TNF/D_3	16.0 ± 3.0
TNF	TNF	14.4 ± 0.3	TNF	TNF	24.2 ± 4.2
IFN-γ	IFN-γ	9.9 ± 0.4	D_3	D_3	7.9 ± 4.8
TNF/IFN-γ	TNF/IFN-γ	29.2 ± 2.6	TNF/D_3	TNF/D_3	63.3 ± 4.5

Cells were cultured for three days with indicated additives (medium alone, 100 U/mL TNF, 100 U/mL IFN-γ, and/or 5 nmol/L D_3), washed, and then cultured another three days with the indicated additives. Results of two experiments are shown.

*Nanomoles H_2O_2 per milligram protein per hour produced in response to 200 nmol/L PMA (mean ± SEM of triplicate samples).

lysozyme was demonstrated in the HL-60 cells with any of the different treatments.

Reactive oxygen species generation. As leukemia cells differentiate to more mature myeloid or monocytoid cells, they gain in their abilities to produce reactive oxygen species such as H_2O_2 and superoxide in response to PMA.[4,5,7] After a three- to four-day incubation with TNF and IFN-γ, there was synergistic enhancement in ability to reduce NBT and produce H_2O_2 (Fig 5C and D). There was a slight increase in H_2O_2 production by the TNF/IFN-γ–treated cells even in the absence of PMA, but it was higher when the cells were stimulated with PMA in the assay (data not shown).

Cell surface antigen changes. After incubation of the HL-60 cells for four to seven days with the TNF and/or IFN-γ, there were increases in the numbers of cells expressing the class II antigen HLA-DR, the monocyte antigen LeuM3, and the C3bi receptor antigen OKM1 (Table 2). In addition, as reflected by the increase in the mean channel of fluorescence, there was an increase in the density of the antigens expressed on the cells.

TNF enhancement of D_3-induced HL-60 cell differentiation. D_3 has been noted to cause monocytoid differentiation of HL-60 cells.[7,37-39] TNF, in an additive and synergistic fashion, enhanced the D_3-induced HL-60 differentiation. This was apparent when assessed by H_2O_2 production and expression of cell surface antigens (Fig 6 and Table 2).

Sequence of addition of the TNF and IFN-γ or D_3. It has been shown in other cell systems that IFN-γ is able to

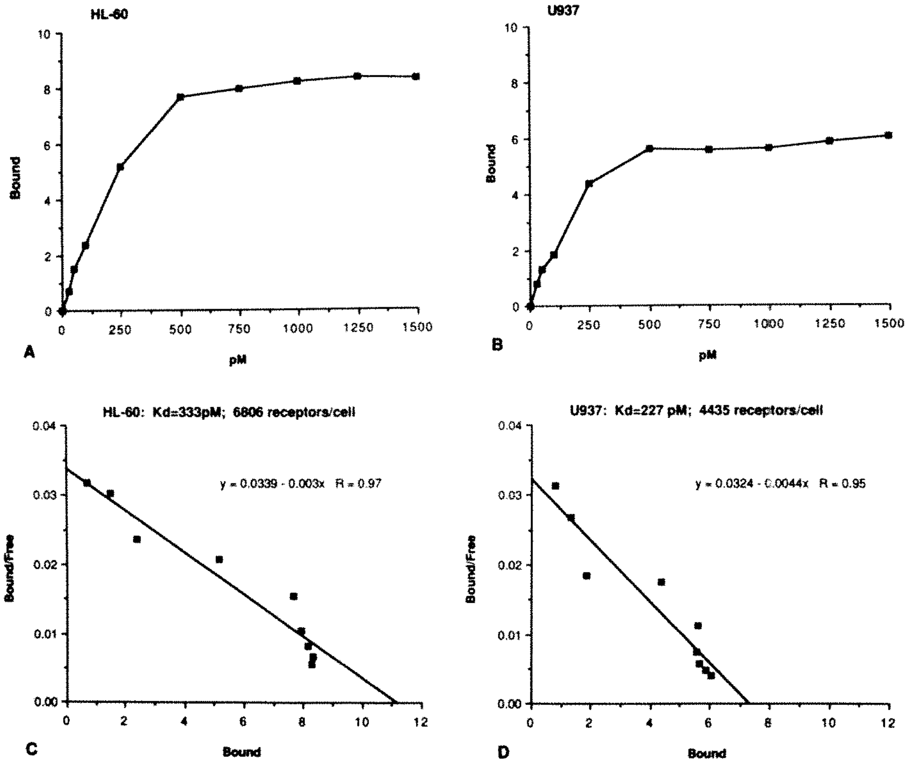

Fig 8. Binding (fmol/10^6 cells) of ^{125}I-labeled TNF to HL-60 and U937 cells as a function of ligand concentration. The binding was for four hours at 0°C. C and D shown Scatchard plots derived from the data in A and B. The data points shown are the means of triplicate samples of a representative experiment from 11 done with HL-60 and seven done with U937 cells.

induce receptors for TNF.[28,40,41] We hypothesized that the synergistic effects seen might be explained by such a mechanism. To test this, a set of experiments was done in which HL-60 cells were incubated for three days with nothing, TNF, D_3, and/or IFN-γ, washed three times, and then assessed for differentiation by measuring their ability to produce H_2O_2 (Table 3). Results demonstrated that the maximal effect was seen when both TNF and IFN-γ or D_3 were together during the six days and that prior treatment with TNF augmented the subsequent response to D_3 but not IFN-γ.

TNF receptors on HL-60 and U937 cells. ^{125}I-labeled TNF bound rapidly, specifically, and reversibly to HL-60 and U937 cells. The binding reached a maximum at about 30 minutes at 23°C and 37°C and at one to two hours at 4°C. But after the 30-minute peak binding at 37°C, cell-bound ^{125}I-labeled TNF reduced progressively over a three-hour period, which suggested the possibility of degradation of the ligand (Fig 7). After incubation for two hours at 4°C with 400 pmol/L ^{125}I-TNF, the subsequent addition of 500 nmol/L unlabeled TNF caused the elution of 52% of the ^{125}I-TNF over a two-hour period at 4°C. Saturation binding done at 4°C for four hours with HL-60 and U937 cells demonstrated saturability, and Scatchard analysis showed comparable binding affinities and numbers of receptors per cell (dissociation constants [K_d] of 226 and 328 pmol/L and receptors per cell of 4,435 and 6,806 for the HL-60 and U937 cells, respectively) (Fig 8). Scatchard plots were linear which suggested single classes of receptors in these cells. Pretreatment of selected cell types with IFN-γ has been shown to increase the numbers of TNF receptors.[28,40,41] However, pretreatment of HL-60 cells for three days with 100 U/mL of IFN-γ or with 5nmol/L D_3 did not appreciably change the affinity or number of TNF receptors (Fig 9).

Differences in responses to TNF could be related to different degrees of TNF internalization and/or degradation. To determine the cellular handling of TNF, HL-60 and U937 cells were incubated with 1,000 pmol/L ^{125}I-labeled TNF at 0°C for two hours, washed twice with cold RPMI-10% FBS, and incubated for varying periods of time at 0° or 37°C. Then the washed cells were treated for five min at 0°C with 0.05 mol/L glycine HCl/0.15 mol/L NaCl buffer (pH 3) to determine the amount internalized. Internalized ^{125}I-labeled TNF is resistant to removal by the low-pH, high-salt buffer, whereas external plasma membrane–bound ligand is removed. Supernatants were collected and analyzed for degraded ligand (^{125}I soluble in 10% trichloroacetic acid). As noted in Fig 10, both cell types rapidly internalized the ^{125}I-labeled TNF at 37°C, and there was some degradation of the TNF at 37°C by both cell types.

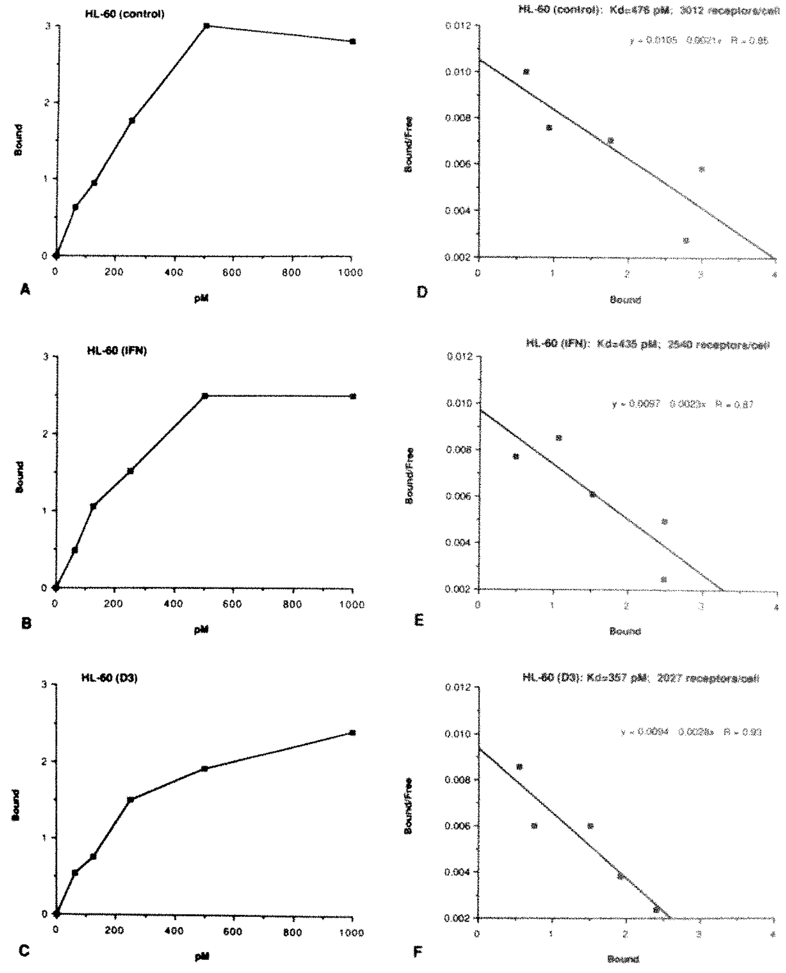

Fig 9. Binding (fmol/10^6 cells) of ^{125}I-labeled TNF to HL-60 cells after three days of culture with 100 U/mL IFN-γ or 5 nmol/L D_3. The binding was for four hours at 0°C. D, E, and F display Scatchard plots derived from the data in A, B, and C. The points are means from triplicate samples from one of two comparable experiments done.

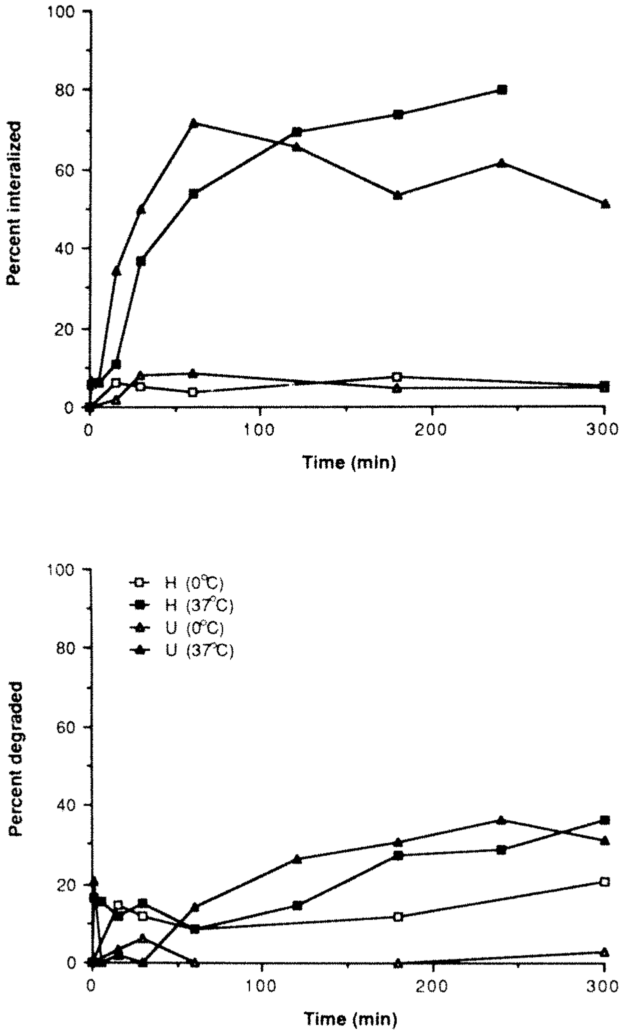

Fig 10. Internalization of ^{125}I-labeled TNF by HL-60 and U937 cells. The cells were first incubated with 1,000 pmol/L ^{125}I-labeled TNF at 0°C, washed, and then incubated for the indicated periods of time at 0 or 37°C. Then washed cells were treated for five minutes at 0°C with 0.05 mol/L glycine HCl/0.15 mol/L NaCl buffer (pH 3) to determine the amount internalized. Internalized ^{125}I-labeled TNF is resistant to removal by the low-pH, high-salt buffer, whereas plasma membrane-bound ligand is removed. Supernatants were collected and analyzed for degraded ligand (^{125}I soluble in 10% trichloroacetic acid). The points represent means of triplicate samples from one of two comparable experiments done.

DISCUSSION

Our results demonstrate that HL-60 and U937 cells have specific, high-affinity receptors for TNF and that TNF acts as a differentiating factor inducing monocytoid differentiation in HL-60 cells and causing cell death in U937 cells. The mechanism by which TNF causes these effects is not known. TNF and IFN-γ, like D$_3$,[7] are able to cause HL-60 cell differentiation with little or no inhibition of proliferation. This is in contradistinction to many of the other differentiating agents such as PMA.[42] It is possible that the TNF acts by inducing other cellular endogenous substances with differentiating capabilities. These would include TNF itself,[25] IFN-γ, or IFN-β_2. In fibroblasts, IFN-β_2 has been noted to be induced by TNF and to influence cell proliferation.[43] In mouse myeloid leukemia M1 cells and human U937 cells, lung conditioned medium and PMA have been shown to cause differentiation and to induce mRNA for IFN-β_2.[44] These possibilities indicate that autocrine factors may play a role in controlling leukemia cell differentiation.

The TNF induction of monocytic surface antigens and nonspecific esterase activity are indicators of monocytic differentiation, whereas the increases in H_2O_2 production and acid phosphatase content are not specific for monocytes/macrophages. Multinucleated macrophages are seen in physiological settings in osteoclasts and in pathological settings in various inflammatory states such as viral, mycobacterial, and fungal infections; foreign body reactions; sarcoidosis; arthritis; and cancer.[45,46] The mechanism by which TNF and IFN-γ cause the multinuclearity in the cells is not known. We have shown before that IFN-γ causes polykaryon formation in nondividing human monocytes by a process of cell fusion.[32] In the rapidly dividing leukemia cells, this is more difficult to determine[7]; it could either be fusion of mononuclear cells or nuclear division without cellular division (endomitosis). The multinucleated cells have some characteristics of osteoclasts, cells thought to be in the mononuclear phagocyte system.[46,47] However, as opposed to osteoclasts, these multinucleated HL-60 cells were not consistently positive for nonspecific esterase, and their acid phosphatase was not tartrate resistant, a typical feature of osteoclasts.[46,47] It is of special interest that TNF or lymphotoxin increases the number of multinucleated osteoclasts in fetal rat bone and increases bone resorption.[48] This indicates that our in vitro observations with human leukemia cell lines may relate to those seen in whole bone in vitro cultures and that these leukemia cells might be useful models for studying these phenomena.

HL-60 and U937 cells have TNF receptors of comparable affinity and number despite differing dramatically in their phenotypic responses to the TNF. TNF was toxic for U937 cells in very small doses, whereas it had little toxic effect on HL-60. The toxicity of TNF for U937 cells made it difficult to do refined characterization of these treated cells. TNF receptors previously described for different cell types vary widely in numbers (a few hundred to 12,000/cell), with the binding affinities in the picomolar range.[14,16,19,27,28,37,38,49,50] Thus, HL-60 and U937 TNF receptors are comparable to those described for other cells. The K$_d$'s for binding of TNF for HL-60 and U937 are somewhat higher than the doses required to induce 50% of the maximal effects (eg, inhibition of ^{3}HTdR incorporation into U937 cells [~6 pmol/L] and induction of nonspecific esterase activity [~50 pmol/L] and peroxide producing ability [~50 pmol/L] in HL-60 cells). Differences in temperatures of the assays as well as length of the cultures may account for some of these differences, or low receptor occupancy may be adequate to generate maximal cellular responses.

The postreceptor transduction and effector mechanisms involved in the TNF ligand/receptor system are not known; there is conflicting evidence regarding the importance of ligand endocytosis with regard to the lytic effects of TNF.[51,52] Our work shows that both U937 and HL-60 cells internalized and degraded ^{125}I-labeled TNF to comparable degrees. In recent work, Peetre et al reported findings similar to some of ours. They showed that TNF inhibited the growth of various human leukemia cell lines and normal marrow CFU-GM and BFU-E.[53] In a wild-type HL-60 cell line whose growth was not inhibited by TNF, the TNF induced monocytic differentiation. As noted in our work here, they showed that leukemia cell TNF receptor number and affinity and internalization/degradation did not predict cellular responses to the TNF.[53]

IFN-γ and D_3 both work additively and/or synergistically in enhancing the differentiating effects of TNF. Both IFN-γ and D_3 mediate their effects on leukemia cell lines through specific receptors.[38,54] Although IFN-γ has been noted to increase the numbers of TNF receptors in other cell types,[28,40,41] neither IFN-γ nor D_3 influenced TNF receptors in the HL-60 cells. Also, pretreatment of the HL-60 cells with IFN-γ or TNF did not enhance the subsequent response to the other, but pretreatment with TNF did increase the response to subsequent incubation with D_3. It is possible that TNF modulates D_3 receptors in causing this.

TNF has been shown to inhibit the growth and differentiation of the murine Friend erythroleukemia cell line.[55,56] TNF also inhibits human CFU-GM, BFU-E, and CFU-GEMM growth in normal bone marrow cells.[57] The differentiating effects of TNF for myeloid cells may play a role in the physiological control of myeloid/monocytic differentiation of hematopoietic cells and may provide a useful approach to the treatment of hematopoietic disorders characterized by defects in myeloid/monocytic differentiation.[58]

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Cytogenetic Evidence for Involvement of B Lymphocytes in Acquired Idiopathic Sideroblastic Anemias

By H. Jeffrey Lawrence, Virginia C. Broudy, R. Ellen Magenis, Susan Olson, Diane Tomar, Sande Barton, John H. Fitchen, and Grover C. Bagby, Jr

We studied the cellular distribution of an unusual chromosomal abnormality, an interstitial deletion of the long arm of chromosome 13, in the peripheral blood lymphocytes of two patients with acquired idiopathic sideroblastic anemia (AISA). We found no metaphases containing the 13q− abnormality in preparations of phytohemagglutinin (PHA)-stimulated lymphocytes from either patient. In both cases, however, some metaphases from Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines contained the clonal karyotypic abnormality. These observations indicate that B lymphocytes but not T cells are expressed as members of the clonal cohort of cells. Our results strongly suggest that the initial pathogenetic events that led to expansion of the 13q− clone occurred in a progenitor cell capable of giving rise to both hematopoietic and B lymphoid cells.
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AMPLE EVIDENCE, using both chromosomal studies and analysis of glucose-6-phosphate dehydrogenase (G6PD) isoenzymes, shows that B lymphocytes are progeny of the neoplastic stem cells in several hematopoietic disorders, including chronic myelocytic leukemia, polycythemia rubra vera, and essential thrombocythemia.[1-4]

Acquired idiopathic sideroblastic anemia (AISA) is a clonal hemopathy classified as one of the myelodysplastic syndromes and characterized by anemia, ringed sideroblasts in the marrow, and minimal disturbance of other cell lines. The level of progenitor cell involvement in this disease is unclear. Using the mosaicism of G6PD isoenzymes in a heterozygous female with AISA, Prchal found that the peripheral blood lymphocytes were clonal and displayed the same isoenzyme as the RBCs, granulocytes, and platelets, suggesting that lymphocytes were part of the neoplastic clone.[5] Raskind et al reported another female, heterozygous for G6PD, with AISA whose Epstein-Barr virus (EBV)-transformed B lymphoblastoid lines were clearly clonal whereas her cultured T cells were not.[6]

We have observed two patients with AISA and an unusual interstitial deletion of chromosome 13. We investigated the involvement of T and B lymphocytes in these two individuals by seeking the 13q− abnormality in phytohemagglutinin (PHA)-stimulated lymphocytes and in EBV-transformed B lymphoblastoid cells. In both cases, we found cytogenetic evidence for partial involvement of B lymphocytes but not T cells.

MATERIALS AND METHODS

Subjects

Patient 1 (R.T.) was a 72-year-old man who came to our hospital for evaluation of a macrocytic anemia. His laboratory evaluation revealed a hematocrit of 32%, mean corpuscular volume (MCV) of 101 μ^3 with normal WBC and platelet counts. Bone marrow aspiration revealed a cellular marrow with erythroid hyperplasia and mildly megaloblastic maturation. Prussian blue stain of the marrow slides revealed numerous ring sideroblasts. A diagnosis of AISA was made. The patient was treated with oral pyridoxine for 6 months but required blood transfusions every month for symptomatic anemia. Testosterone therapy by intramuscular depot produced an improvement in his anemia, during which time he required no transfusions.

Patient 2 (A.S.) is a 62-year-old man with a 4-year history of anemia. Laboratory evaluation included a hematocrit of 28% with a reticulocyte count of 1%, and normal WBC and platelet counts. Bone marrow aspiration showed a hypercellular marrow with megaloblastoid erythropoiesis. Prussian blue stain revealed many ringed sideroblasts. A diagnosis of AISA was made. Trials of pyridoxine and androgens were ineffective.

Karyotypic Analysis

Heparinized (1,000 U/mL) bone marrow (0.1 mL) was incubated overnight at 37°C in 5 mL RPMI 1640 medium with 10% fetal calf serum (FCS) and 1% gentamicin (50 μg/mL). Cells were harvested, and slides were made. Chromosome preparations were G-banded for chromosome analysis.[7] Approximately 20 metaphases were analyzed.

PHA-stimulated peripheral blood. Heparinized whole blood (0.5 mL) was inoculated into culture tubes containing 5 mL RPMI 1640 medium with 10% FCS gentamicin, 2% glutamine, and 1% PHA (Wellcome, Research Triangle Park, North Carolina). Cells were harvested according to established procedures.[8] Slides were prepared and stained using the G-banding technique.[9] Approximately 20 to 40 metaphases were examined.

EBV-transformed peripheral blood lymphocytes. Peripheral blood mononuclear cells (PBMCs) were obtained from heparinized venous blood by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density-gradient centrifugation.[10] Preferential transformation of B lymphocytes in the PBMCs was accomplished by adding EBV (100 μL/mL of EBV suspension containing 1×10^7 transforming units/mL) to suspensions of PBMCs (1×10^6 cells/mL) in RPMI 1640 with 20% FCS and 2% glutamine.[11-13] Cultures were harvested at 96 hours, slides were made, and preparations were stained with quinacrine.[14] Between 100 and 150 cells were analyzed and photographed using a Zeiss photoscope III. In cells demonstrating the deleted 13q, fluorescent variant regions were observed to determine whether the deletion was consistently in the same homolog.

RESULTS

Cytogenetic studies of unstimulated bone marrow cells were performed in both cases. Metaphases from both cases

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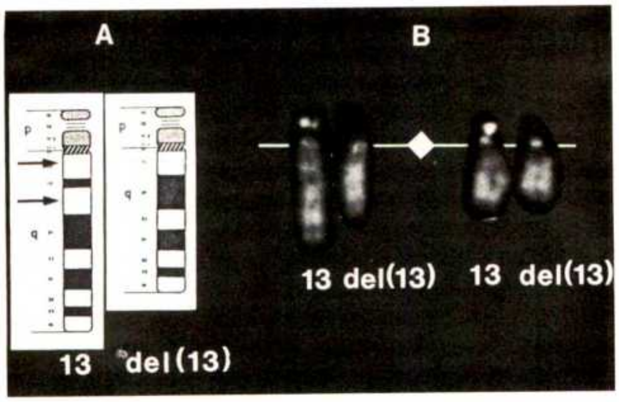

Fig 1. (A) ISCN (1985) ideograms.[19] Left 13 is normal; arrows indicate deletion breakpoints. Right 13 illustrates banding pattern of abnormal 13. (B) Representative quinacrine-stained chromosomes 13 from marrow cells of patient 2. Left chromosomes 13 of each pair are normal. Right chromosomes 13 have an interstitial deletion, bands q12 → q14. Fluorescent variant regions of the chromosomes were consistent with the deletion occurring in the same homolog in each cell examined. The normal 13 has bright short arms, medium-length stalk region, and bright medium-size satellites. The deleted 13 has bright short arms, long stalks, and small, pale satellites.

displayed an interstitial deletion of chromosome 13 with or without other karyotypic abnormality. In case 1, six of 18 cells displayed a 46,XY,del(13)(q11;q21) karyotype. In case 2, 21 of 21 cells had a del(13)(q12;q14) (Fig 1). In addition, three of 21 metaphases were missing a number 21 chromosome.

Cytogenetic evaluation of PHA-stimulated lymphocytes was carried out on blood samples from both patients to assess whether T cells were involved in the neoplastic clone. In both cases, only normal metaphases were seen (Table 1). To assess B cell involvement, karyotypic analysis of EBV-transformed lymphocytes was performed on blood samples from both patients. In each case, some metaphases containing the 13q interstitial deletion were identified. Comparison of fluorescent chromosome 13 heteromorphisms was consistent with the deletion involving the same homolog in each cell examined (Fig 1).

Table 1. Frequency of Abnormal Metaphases in Marrow Cells and Peripheral Blood Lymphocytes

Patient	BM*	PHA†	EBV‡
1	6/18§	0/40	3/100
2	21/21	0/20	20/151

*BM = unstimulated bone marrow cells.[7]

†PHA = stimulated peripheral lymphocytes.[8,9]

‡EBV = transformed peripheral lymphocytes.[10-14]

§Number of metaphases containing 13 q− deletion/total number of metaphases examined.

DISCUSSION

These cases are, to the best of our knowledge, the first examples of AISA for which there is cytogenetic evidence of involvement of lymphocytes. Raskind et al were able to demonstrate B cell involvement in a case of AISA using G6PD isoenzymes but not by karyotypic analysis.[6] They hypothesized a two-step mutational process, the first involving B lymphocytes as well as other hematopoietic cell lines and manifested by a single G6PD isoenzyme in all involved cells, and the second with more restricted involvement of nonlymphoid hematopoietic cells and manifested by a clonal chromosomal abnormality.

Our results, unlike those of Prchal, have not documented that T lymphocytes are part of the clonal disorder. Our findings are consistent with most observations in other clonal hematologic diseases such as polycythemia rubra vera and chronic myelogenous leukemia,[2-5] although T cell involvement has been suggested by some studies.[15] In our cases, the extent of T lymphocyte involvement may have been small and might have been detected if more metaphases had been available for analysis.

Other evidence for involvement of lymphocytes in AISA and in myelodysplastic syndromes is less clear-cut. A case of acute lymphocytic leukemia (ALL) has been reported in a patient with AISA, but the diagnosis of ALL was made primarily by morphological criteria and not by immunophenotyping.[16] Functional abnormalities of lymphocytes in myelodysplasia have been reported by other researchers.[17,18] One curious observation was a lack of receptors for EBV with resultant resistance to in vitro infection with the virus. EBV-transformed lymphoblastoid cell lines were easily established from peripheral blood of both our patients.

In summary, our studies have provided clear cytogenetic evidence of partial involvement of B lymphocytes, but not T cells, in the neoplastic clone in AISA. Whether this pattern of lymphocytic involvement is specific for patients with the 13q− abnormality awaits further study of cases of AISA with different karyotypic abnormalities.

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Carrier Testing in Hemophilia B With an Immunoassay That Distinguishes a Prevalent Factor IX Dimorphism

By Kenneth J. Smith, Arthur R. Thompson, Brad A. McMullen, Dan Frazier, Shu Wha Lin, Darrell Stafford, Walter Kisiel, Stephen N. Thibodeau, Shi-Han Chen, and Leslie F. Smith

Immunoassays with a monoclonal antibody (A-1) detect a prevalent dimorphism in plasma coagulation factor IX. The antibody was shown to react with a dimorphic segment of the normal factor IX sequence as follows. First, A-1 bound to isolated activation peptide (residues 146 through 180) prepared from activated factor IX from a normal plasma pool. Second, binding of recombinant factor IXs with A-1 or factor IX from normal individuals was strong only when they had Threonine (Thr) at position 148; factor IXs with the Alanine (Ala) allele at that position were far less reactive. Third, immunoblot reactivity of *Escherichia coli* fusion proteins containing known segments of the factor IX sequence restricted the epitope to residues 147 through 153. In 120 hemophilia B pedigrees, the Ala immunoassay allele frequency was 0.19 and did not differ from the Ala frequency in normal males. In 22 of 49 families, immunoassay testing was informative for classification of obligate or possible carriers. In one large family, 4 obligate carriers were heterozygous for the dimorphism and 3 of their 7 daughters were classified as carriers. In other families, when the affected member had <1 nmol/L factor IX antigen, heterozygosity for Thr/Ala alleles excluded the carrier state even when DNA studies were not informative. Strong linkage disequilibrium of Thr/Ala alleles with the common *TaqI* DNA polymorphism was found. Nineteen of 75 normal and hemophilic factor IX genes had the 1.3-kilobase (kb) fragment and coded for the Ala allele; the rest had the 1.8-kb fragment and coded for Thr. In selected families, the A-1 immunoassay is an inexpensive and rapid method to confirm and supplement restriction fragment length polymorphism analyses of DNA for carrier testing.

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DEFICIENCY of factor IX clotting activity causes hemophilia B, a hereditary bleeding disorder with clinical manifestations indistinguishable from those seen with factor VIII deficiency. The molecular defects leading to hemophilia B are heterogenous.[1,2] Single amino acid substitutions,[3-6] a donor splice junction defect,[7] and gene deletions of various size[8,9] have been described.

In kindreds with readily measurable dysfunctional plasma factor IX, the expression of excess antigen as compared with clotting activity has been used to detect carriers.[10,11] In some kindreds, the abnormal factor IX expressed differs in mol wt from native factor IX and can be distinguished from normal factor IX on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE),[4,5] but carrier detection using this technique has not been described.

Accuracy of carrier testing based on expression of the abnormal gene is limited by random inactivation of X chromosomes in female hepatic cells and by the wide distribution of factor IX antigen and activity among normal subjects. In contrast, the accuracy of family studies for carrier testing using intragenic restriction fragment length polymorphisms (RFLPs) is limited only by technical or sample errors or by unlikely recombination in the family tested.[12] Quantitative cDNA hybridization studies may also detect gene deletions in potential carriers.[13] RFLP analyses, although well established, are time-consuming and expensive.

In 1985, McGraw et al described a prevalent DNA and protein polymorphism at amino acid residue 148 in normal and hemophilic factor IX.[14] They predicted that the threonine (Thr) allele was more prevalent than alanine (Ala) in the general population. Recently, carrier testing by oligonucleotide probes specific for the Thr or Ala codons has been described.[15] The codon for the Ala allele was present at a frequency of 0.33 (95% confidence interval, 0.21 to 0.45) in the normal population. Two independent reports have shown that monoclonal antibody immunoassays can detect a dimorphism in plasma factor IX.[16-18] The A-1 antibody distinguishes factor IX alleles with a frequency of 0.25 for the less frequent form (95% confidence interval of 0.14 to 0.36).[17]

The current study demonstrates the specificity of the A-1 monoclonal antibody for an epitope near the amino terminus of the activation peptide of factor IX. This epitope includes residue 148, which is dimorphic. The A-1 antibody binds well to factor IX with Thr at residue 148 and weakly to factor IX with Ala at that site. Thus, the dimorphism is the basis for immunoassay reactivity. Heterozygous women show intermediate immunoassay reactivity and, in families in which abnormal factor IX is expressed, the assay can be used as a rapid initial screen to aid in hemophilia B carrier testing.

MATERIALS AND METHODS

Materials. Sephadex G-100, diethylaminoethyl (DEAE)-Sepharose CL-6B, activated CH-Sepharose 4B, and electrophoresis mol-wt standards were from Pharmacia, Piscataway, NJ. Acetonitrile and benzamidine were from Aldrich Chemical, Milwaukee, WI. Affigel-10, acrylamide, *bis*-acrylamide, ammonium persulfate, and N, N, N^1, N^1-tetramethylethylenediamine were from BioRad, Rich-

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mond, CA. Low-mol-wt electrophoresis standards were from Pierce, Rockford, IL. *Taq*I was from New England Biolabs, Beverly, MA or BRL, Bethesda, MD. Peroxidase was from Boehringer Mannheim Biochemicals, Indianapolis, and *o*-phenylenediamine was from Sigma Chemical, St Louis.

Factor IX preparations. Factor IX was partially purified from plasma pooled from 20 to 30 patients undergoing therapeutic plasmapheresis.[19] In the final purification step, 5 to 20 mg factor IX in 0.05 mol/L NaCl/0.05 mol/L Tris-HCl (pH 7.2)/10 mmol/L benzamidine HCl/20 mmol/L $MgCl_2$ was passed over a 1 × 8-cm column of A-7 antibody (anti-light chain of factor IX_a)[20] coupled to Affigel-10 at a concentration of antibody 3 mg/mL gel. The column was washed with three column volumes of 1.0 mol/L NaCl/0.02 mol/L Tris-HCl (pH 7.2)/20 mmol/L $MgCl_2$/1 mmol/L benzamidine HCl and then eluted with 20 mmol/L EDTA in 0.15 mol/L NaCl/0.02 mol/L Tris-HCl (pH 7.2)/1 mmol/L benzamidine-HCl. Factor IX from individual male patients undergoing plasmapheresis was purified by batch adsorption and elution from an ion-exchange matrix, as previously described,[21] with the modification that DEAE-Sepharose was used instead of DEAE-Sephadex. The DEAE-Sepharose eluate was precipitated with 65% saturated ammonium sulfate and then dissolved in 0.15 mol/L NaCl/0.02 mol/L Tris-HCl (pH 7.2)/10 mmol/L benzamidine-HCl/20 mmol/L $MgCl_2$ before isolation of factor IX by monoclonal antibody immunoaffinity chromatography. Factor IX preparations were homogenous by SDS-PAGE.

Tissue culture supernatants with recombinant factor IX were provided by Dr George Brownlee, Cambridge, England,[22] Dr Sharon Busbee, Zymogenetics, Seattle,[23] Dr Charles Shoemaker, Genetics Institute, Cambridge, MA,[24] and Dr Henri De LaSalle, Transgene S.A., Strasbourg, France.[25] Immunoassays were done on tissue culture supernatants from Dr Brownlee and Dr De LaSalle that had been previously lyophilized. Otherwise, samples had been stored at −70°C.

Factor IX activation and activation peptide isolation. Factor IX (0.5 to 1.0 mg/mL), prepared from pooled plasma or individual males was activated for 4 hours with solid-phase crude factor XIa[26] in 0.1 mol/L NaCl/0.05 mol/L Tris-HCR (pH 8)/10 mmol/L $CaCl_2$. Factor XIa was coupled to activated CH-Sepharose at a concentration of 5 to 10 mg/mL gel. The volume of factor XIa-Sepharose added was 5% of the factor IX solution. After addition of EDTA to a final concentration of 20 mmol/L, solid-phase XIa was removed by centrifugation and the supernatant was dialyzed against 0.1 mol/L NH_4HCO_3 and lyophilized. In one preparation of factor IX from a male individual, the activation peptide was purified from activated factor IX by gel filtration,[27] but for three subsequent individuals, purification was done by reverse-phase chromatography on an Altex C-3 column (Beckman Laboratories, Berkeley, CA) with a Waters 6000A Solvent delivery system (Waters Associates, Milford, MA). In this procedure, lyophilized preparations (1 to 2 mg) were dissolved in 0.1% trifluoracetic acid (TFA). After sample application, the column was washed for 2 minutes with 0.1% TFA. The activation peptide was then eluted with a gradient of 0% to 60% acetonitrile developed in 0.1% TFA over 20 minutes. Absorbance was read at 230 nm.

For larger scale isolation of activation peptide from pooled plasma, 100 mg factor IX was activated with 10 mL factor XIa-Sepharose and then lyophilized after dialysis in 0.1 mol/L NH_4HCO_3. The activation peptide was separated on Sephadex G-100 in 3 mol/L urea with 1% formic acid. Late eluting peaks with absorbance at 280 nm were pooled, dialyzed against 0.1 mol/L NH_4HCO_3, and lyophilized again. One-milligram to 4-mg samples of activation peptide were dissolved in 0.1% TFA and injected for reverse-phase high-performance liquid chromatography (HPLC). The gradient for activation peptide purification used 0.1% TFA (buffer A) and 0.08% TFA in acetonitrile (buffer B). The pumps ran buffer A for 2 minutes, 0% to 30% buffer B from 2 to 12 minutes, then 30% to 45% buffer B from 12 to 22 minutes, and finally 45% to 69% buffer B from 22 to 30 minutes.

Amino acid analyses on samples of activation peptide were performed on a Durrum DC-1A cation-exchange resin with detection by orthophthaldehyde. Methods for amino terminal sequence analysis of activation peptide and minor C-terminal factor IX fragments were as previously reported.[28]

Immunassays. Factor IX antigen was determined using immunoradiometric assays. The A-1 antibody or the A-5 antibody (anti-heavy chain of factor IXa) were used to coat microtiter plates and bind factor IX samples. Bound factor IX was quantitated with radioiodinated A-7 antibody (anti-light chain of factor IXa).[17] In some experiments, the labeled antibody was a fractionated polyclonal preparation.[29] Another monoclonal antibody to an epitope in the heavy chain of IXa[30] could be substituted for solid-phase A-5.

Immunoreactive activation peptide and factor IX were also tested in an antibody binding inhibition assay. Serial twofold dilutions of factor IX or test samples in 0.15 mol/L NaCl/0.02 mol/L Tris-HCl (pH 7.2), with 1 mg/mL ovalbumin were mixed with peroxidase conjugated[31] A-1 antibody for 2 hours at room temperature. The mixture of radioiodinated antibody (100 μL) and antigen was then added in triplicate to microtiter wells previously coated with factor IX at a concentration of 1 μg/mL. After 2 hours at 37°C, bound antibody was quantitated with addition of peroxidase substrate (*o*-pheynlenediamine in 0.1 mol/L sodium citrate, pH 4.5).

Reaction of factor IX fusion proteins with monoclonal antibody A-1. The T7 expression system described by Studier and Moffat[32] was used for expression of the different fragments of human factor IX cDNA.[14] The fusion proteins were produced as insoluble proteins in *Escherichia coli* and were rendered soluble by boiling in a buffer containing SDS and 2-mercaptoethanol. An aliquot of each solubilized protein was applied to Laemmli[33] gels with 4% acrylamide in stacking gels and 10% acrylamide in running gels for electrophoresis. The electrophoretically fractionated proteins were transferred to a nitrocellulose membrane and reacted with either ^{125}I-A-1 or with nonradioactive A-1 antibody followed by ^{125}I-protein A (ICN, Cleveland).

As previously described,[34] a λ gt11 expression library was constructed containing random, small DNase I fragments of the coding region of a human factor IX cDNA. The recombinant phage were screened for immunoreactivity, using A-1 monoclonal antibody and ^{125}I-Protein A or ^{125}I-A-1 prepared by chloramine-T technique.[17]

Ten-milliliter aliquots of the purified recombinant phage were grown from the pure phage stocks, the DNA was isolated,[35] and the *Eco*RI fragment containing factor IX cDNA was ligated direcly into the single-strand phage M13 mp18[36] for dideoxy sequencing using 35-S dATP.[37]

Immunoblotting of factors IX and IXa with A-1. Two- to 5-μg samples of factor IX or factor IXa were applied to lanes of Laemmli gels and electrophoresed prior to transfer to nitrocellulose. After nonspecific binding was blocked by soaking the nitrocellulose in 5% nonfat dry milk in 0.15 mol/L NaCl/0.02 mol/L Tris-HCl (pH 7.2) with 0.2% sodium azide, radioiodinated monoclonal antibodies with a specific activity of 2 μCi/μg were diluted to 10^6 cpm/mL in the same solution for reaction with nitrocellulose for 2 hours at room temperature. Nitrocellulose sheets were washed in buffer without 5% milk solids three times and then exposed overnight for radioautography.

Studies on human subjects. Plasma from patients undergoing therapeutic plasmapheresis was obtained from United Blood Services of Albuquerque. Informed consent for blood samples was obtained as approved by the University of Washington's Human Subjects Review Committee. One hundred twenty hemophilia B pedigrees with >2 nmol/L factor IX antigen concentration were tested by immunoassay, as were females from 49 families. EDTA plasma samples and DNA from 39 unrelated normal males from families with hereditary disorders other than hemophilia B were tested for immunoassay alleles and *Taq*I RFLPs using a factor IX cDNA probe.[38] *Taq*I RFLPs and immunoassays were also compared for affected members in 28 hemophilia B kindreds.

RESULTS

A-1 reactivity with factor IX activation peptide. Factor IX activation peptide was purified from factor IX obtained from pooled plasma and from 2- to 3-L amounts of plasma from male patients undergoing therapeutic plasmapheresis. The late eluting peak from gel filtration of factor IXa preparations from an individual male plasma and from pooled plasma were resolved into three peaks (Fig 1A) when analyzed on reverse-phase HPLC. The first two peaks (I and II, Fig 1A) did not stain with Coomassie blue but were readily stained with periodic acid-Schiff (PAS) reagent[39] and showed identical migration on SDS-PAGE (Fig 1B, gel 1). The third peak (III, Fig 1A) did not stain with PAS but could be stained with Coomassie blue (Fig 1B, gels 2 and 3). PAS-positive peaks resolved by HPLC did not migrate at the expected position for factor IX activation peptide on SDS-PAGE using either Laemmli gels (pH 8.8) or Weber Osborn gels (pH 7.0).[40] When the percentage of acrylamide in gels was 12.5%, the apparent migration of the glycopeptide was 30,000 mol wt, whereas on 15% gels, the apparent migration was 25,000 mol wt. The gel migration and staining properties of activation peptide differ from those previously described.[27]

Samples obtained by HPLC were analyzed by N-terminal sequence analysis and for amino acid content. PAS-positive peaks (I and II) obtained on HPLC of activation peptide from pooled plasma were identical in N-terminal sequence and amino acid content. The first eight residues for both peaks gave the sequence Ala-Glu-(Thr/Ala)-Val-Phe-Pro-Asp-Val, corresponding to residues 146 through 153 in factor IX.

Observed and (expected) values for amino acid content of peak II were as follows: Asp 7.3 (7), Thr 5.6 (6), Ser 2.6 (3), Glue 5.1 (5), Ala 2.2 (2), Val 3.0 (3), Ile 1.9 (2), Leu 1.1 (1), Tyr 0.8 (1), Phe 3.7 (3), and Arg 1.1 (1). Proline was not detected with orthopthlaldehyde in the method used for amino acid analysis. The expected values given are for the more prevalent sequence containing Thr rather than Ala and Thr and Ser content is corrected for loss on hydrolysis. The glycopeptide from either HPLC peaks I or II was able to inhibit the binding of the A-1 antibody to factor IX immobilized on microtiter wells (Fig 2). These peptides did not inhibit the binding of other monoclonal antibodies to immobilized factor IX (data not shown).

The peak with the greatest retention time on reverse-phase HPLC (peptide III, Fig 1A; Fig 1B, gels 2 and 3) did not inhibit the binding of the A-1 antibody to solid-phase factor IX and did not stain with PAS. Peptides in this peak were a minor component of the activation peptide preparation from gel filtration of IXa, since their N-terminal residues were not detected in N-terminal amino acid sequence analysis. These peptides were probably cleaved from the heavy chain of factor IXa by contaminating proteases in the crude celite eluant (amino acid sequence data not shown).

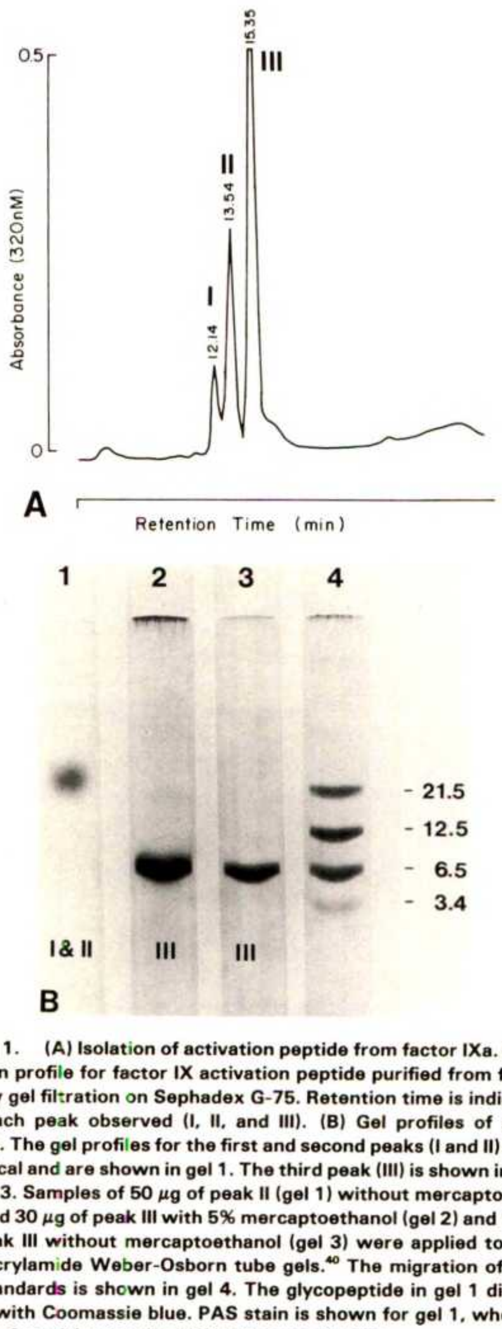

Fig 1. (A) Isolation of activation peptide from factor IXa. HPLC elution profile for factor IX activation peptide purified from factor IXa by gel filtration on Sephadex G-75. Retention time is indicated for each peak observed (I, II, and III). (B) Gel profiles of HPLC peaks. The gel profiles for the first and second peaks (I and II) were identical and are shown in gel 1. The third peak (III) is shown in gels 2 and 3. Samples of 50 μg of peak II (gel 1) without mercaptoethanol and 30 μg of peak III with 5% mercaptoethanol (gel 2) and 15 μg of peak III without mercaptoethanol (gel 3) were applied to 15% polyacrylamide Weber-Osborn tube gels.[40] The migration of mol-wt standards is shown in gel 4. The glycopeptide in gel 1 did not stain with Coomassie blue. PAS stain is shown for gel 1, whereas gels 2, 3, and 4 are stained with Coomassie blue.

A-1 reaction with activation peptide was further tested with Western blotting. Native factor IX and an activation intermediate of factor IX (heavy chain of IXa and factor IXa activation peptide) were recognized by the A-1 antibody, but the heavy chain of factor IXa was not (Fig 3). Antibody binding was not seen in the position expected for the activation peptide itself, although tritium label could be detected on nitrocellulose in the expected migration position for activation peptide after SDS-PAGE and electrophoretic

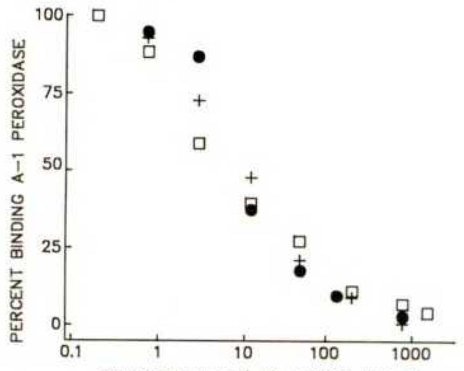

Fig 2. Inhibition of peroxidase-conjugated A-1 antibody by factor IX, factor IXa, and activation peptide. Factor IX (●) and activation peptide (□) and factor IXa (+) in 0.15 mol/L NaCl/0.02 mol/L Tris-HCl with 1 mg/mL ovalbumin were added to a solution of peroxidase conjugated monoclonal antibody at ~10 nmol/L concentration. The mixture was incubated for 2 hours at room temperature and then added to microtiter wells coated with factor IX. After 2 hours at 37°C, bound antibody was quantitated with addition of *o*-phenylenediamine in 0.1 mol/L sodium citrate, pH 4.5. The concentration of activation peptide was quantitated by an extinction coefficient determined by amino acid analysis of a sample of known absorbance at 230 nm. Factor IX and factor IXa were quantitated using an extinction coefficient of 13.3 for a 1% solution at 280 nm.[27]

transfer of tritiated factor IXa (data not shown). When ^{125}I-staphylococcal protein A and nonradioactive A-1 had been used for radioautography in previous experiments,[41] there was greater background binding, erroneously interpreted as A-1 binding to the heavy chain of factor IXa.

Specificity of A-1 for the Thr allele. Amino acid sequence analysis on isolated activation peptide prepared from pooled plasma showed both Thr and Ala at position 3. The ratio of Thr to Ala of position 3 was calculated as 3.8 to 1 based on a repetitive yield of 21% of the expected Ala yield at turn 3 given yields of Val at turns 4 and 8. This result is similar to the predicted prevalence of 3 to 1 of Thr over Ala in pooled plasma from which the factor IX was derived.[17] Partially sequenced factor IX activation peptide from each of three males with the more prevalent immunoassay allele (with strong A-1 binding) had only Thr at position 148, and one sample with the less frequent allele in the immunoassay (weak A-1 binding) had only Ala at this position (Table 1).

Factor IX produced by recombinant DNA technology was tested in a two-antibody immunoassay system using the A-1 antibody or the A-5 antibody (anti-heavy chain of factor IXa), as the solid-phase moiety as shown in Table 1 (samples 6 through 9). The samples expressing Ala at position 148 had little reaction with A-1 antibody in immunoassays.

Studies with fusion proteins. The region of factor IX recognized by A-1 was also defined by its reaction with specific fragments of human factor IX expressed in *E coli* under control of the T7 gene 10 promoter and reaction with a series of overlapping random fragments of human factor IX cDNA expressed in λ gt11 as fusion proteins to β-galactosidase. The specific fragments made under the control of the T7 promoter included amino acid residues −3 through 180*, 1 through 49, 50 through 111, 112 through 142, 116 through 180*, 147 through 180*, 180 through 265, 266 through 324, 335 through 415 and −3 through 415* (fragments marked with an asterisk reacted with A-1). Reactivity with the specific fragments limit the antigenic determinant to the activation peptide. To define the epitope further, the random fragment library in λ gt11 was screened with A-1, positive plaques were purified, and the DNA insert was sequenced. Approximately 1 in 1,000 clones was reactive with A-1.

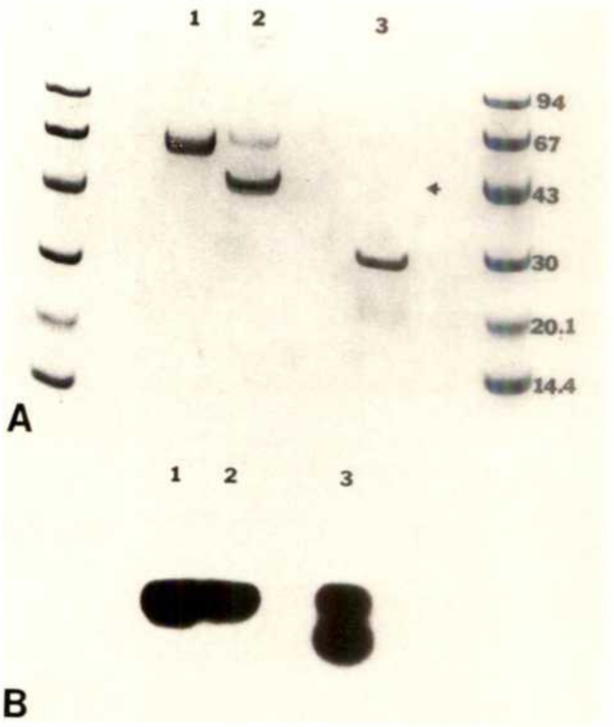

Fig 3. Immunoblotting of factor IX and activated factor IX with A-1. In panel A, 2-μg samples of factor IX (lane 1) and factor IXa (lanes 2 and 3) were analyzed by SDS-PAGE.[40] A slab gel stained with Coomassie blue (panel A) shows migration of native factor IX and factor IXa without mercaptoethanol (lanes 1 and 2, respectively), and migration of factor IXa with 5% mercaptoethanol (lane 3). The 44-kd activation intermediate is indicated (arrow). Mol-wt standards are on the left and right margins of the gel. In panel B, samples in an identical gel were transferred to nitrocellulose and detected with ^{125}I-A-1. No low-mol-wt cleavage products were identified in lane 2, whereas an activation intermediate (heavy chain of factor IXa and covalently linked activation peptide) is apparent ~44 kd. No reactivity with activation peptide itself or the heavy and light chains of factor IXa was found.

One λ gt11 insert was sequenced and found to extend from base 352 to base 460 of the zymogen cDNA coding sequence (17,767 to 17,797 and 20,363 to 20,480 in the numbering of Yoshitaki).[42] This insert codes for a peptide extending from Ala 118 to Val 153. A second nucleotide cloned was sequenced and was identical except for being one base shorter. The overlap between these fragments and a reactive fragment of the specific T7 gene 10 promoter system is amino acids 147 through 153 of the activation peptide, including residue 148 with the polymorphism (Fig 4). The

Table 1. Factor IX Antigen Concentration (nmol/L) by Immunoassay

Sample	Immunoassay A-1, A-7*	Immunoassay A-5, A-7*	Amino Acid at Residue 148	Codon 20,422–20,424
1. Pooled plasma	67	89	Thr/Ala (4:1)	—
2. Male donor A	127	131	Thr	—
3. Male donor B	91	86	Thr	—
4. Male donor C	93	83	Thr	—
5. Male donor D	9	90	Ala	—
6. rDNA IX[22]	<0.09	1.1	—	GCT
7. rDNA IX[24]	<0.45	9.1	—	GCT
8. rDNA IX[23]	<0.18	8.6	—	GCT
9. rDNA IX[25]	7.4	7.7	—	ACT

Samples of recombinant factor IX, pooled normal plasma, or plasma from male donors undergoing therapeutic plasmapheresis were tested in an immunoassay system in which either the A-1 (anti-activation peptide) or A-5 (anti-factor IXa heavy chain) was used as the solid-phase antibody in a two-antibody immunoradiometric assay (described in text). After incubation of diluted plasma or tissue culture supernatant samples in the wells, bound antibody was detected with radioiodinated A-7 antibody (A-7*). The radio iodinated antibody in the two antibody assays is indicated by an asterisk. Control media with 10% fetal calf serum had <0.09 nmol/L factor IX antigen content. The factor IX concentration in pooled plasma is assumed to be 89 nmol/L. Plasma levels of factor IX measured with the A-1, A-7* immunoassay system were determined by comparison with standard curve generated by dilution of plasma factor IX from a single male with the Thr allele. The codon numbering is that of Yoshitaki et al.[42] Reference numbers are given for the source of recombinant DNA factor IX samples.

cDNA expressed had the ACT codon at bases 20,422 through 20,424 for Thr, accounting for reactivity of its expressed fragments with A-1.[14]

Human subjects. Plasma samples from 120 kindreds with hemophilia B and >2 nmol/L factor IX antigen concentration (>2.0 U/dL or >2% of normal) were tested by immunoassay to determine the number of individuals expressing Thr or Ala. The frequency of the Ala allele was 0.19 (95% confidence interval, 0.11 to 0.28). This did not differ from the frequency of 0.25 in normal males (95% confidence interval, 0.14 to 0.36). In 12 kindred in which more than one affected individual was available for testing, the immunoassay Thr/Ala classification was consistent for the abnormal gene product within each family.

The association of the *Taq*I 1.8-kb fragment with the Thr allele and the 1.3-kb allele with the Ala allele by immunoassay result was 100% in affected members from 28 tested kindreds with hemophilia B. The association of the two alleles with these *Taq*I fragments in 39 unrelated normal males was also 100% (Table 2), indicating strong linkage disequilibrium of the DNA and protein polymorphisms.

Of the 114 females from 48 additional hemophilia B families, 53 were from 27 families in which immunoassays were not informative for carrier detection. In the other 21 families, immunoassays in 40 of 61 were informative. These include 27 women who were Thr/Ala heterozygotes and nine women with a homozygous phenotype different from the immunoassay allele expressed in the hemophilic gene product. In four families, the sons of obligate carriers had <1 nmol/L factor IX antigen. The carrier state could be excluded in four women in three of these families because they expressed nearly equal amounts of the Thr/Ala alleles and had normal factor IX antigen levels. In two of these families, RFLP analyses of DNA were not informative (data not shown).

Twenty-four samples from the large pedigree shown in Fig 5 and Table 3 were tested by immunoassay for the Thr and Ala alleles. Four obligate carriers (III-5, III-6, III-7, and III-8) had intermediate reactivity to A-1, indicating that they were heterozygous for the Thr and Ala alleles. The hemophilic gene was associated with the Thr allele in all five affected family members tested (II-1, III-10, IV-4, IV-8, IV-12). All four nonaffected males who were children of the four heterozygous carriers had the Ala allele (IV-6, IV-7, IV-10, IV-11). The carrier status of seven possible carriers could be predicted in generation IV by the immunoassay result. Four tested as noncarriers (IV-5, IV-9, IV-16, IV-17), and three were predicted to be carriers (IV-13, IV-14, IV-15) (Table 3, Fig 5).

Carrier testing was confirmed by restriction fragment length polymorphisms. Leukocyte DNA was obtained from two patients (IV-8 and IV-12) and five other family members (III-5, III-7, and III-7a and IV-13, IV-14). DNA was digested with *Taq*I and hybridyzed to a cDNA probe. The

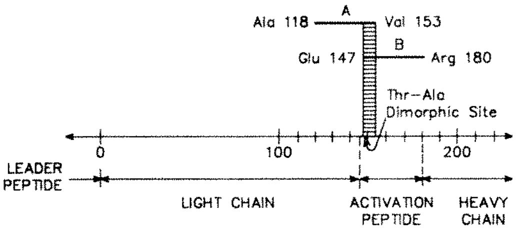

Fig 4. Diagram of A-1 epitope. Factor IX regions encoded by two immunoreactive clones: (A) a λ gt11 insert; (B) a fragment from the T7 expression system. The overlap region (shaded) must include part of the epitope recognized by A-1.

Table 2. Association of *Taq*I Polymorphic Pattern and Immunoassay Alleles in Normal (and Hemophilic) Subjects

*Taq*I Fragments (kb)	Thr	Ala	Total
1.8	28 (24)	0	52
1.3	0	11 (4)	15

Thirty-nine unrelated males and 28 patients from separate hemophilic kindreds were tested for Thr/Ala and *Taq*I alleles. Linkage disequilibrium exists between less common *Taq*I 1.3-kb and Ala alleles and the more common *Taq*I 1.8-kb and Thr alleles.

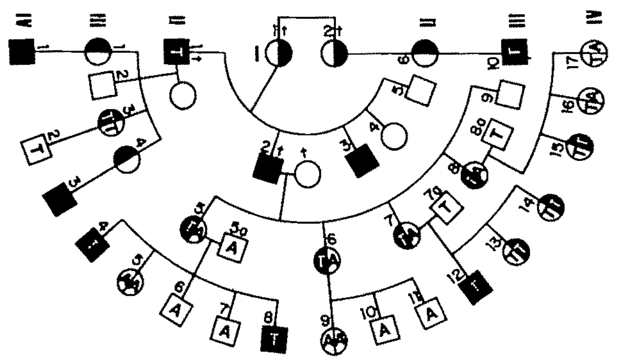

Fig 5. Family study with A-1 (Thr/Ala). Immunoassay alleles (T, Thr; A, Ala) are shown for the family members tested (Table 2). Affected males and obligate carriers or carriers assigned by immunoassay are shown with black shading.

two affected males and the one father (III-7a) examined had the more frequent 1.8-kb band whereas the obligate carriers (III-5, III-7) were heterozygous with 1.8-kb and 1.3-kb bands. Both potential carriers (IV-13 and IV-14) had the 1.8-kb fragment alone, indicating that they had inherited their mother's hemophilic factor IX gene, confirming results of the immunoassay studies.

DISCUSSION

McGraw et al presented data on a cDNA, genomic DNA, and protein sequence dimorphism at residue 148 in the activation peptide.[14] They found that the cDNA from a plasmid library and genomic DNA prepared by restriction endonuclease digestion of genomic DNA obtained from a patient with hemophilia B both coded for Thr. Amino-terminal sequence of activation peptides from normal pooled plasma and a female showed both Thr and Ala at position 148, whereas factor IX from a normal male donor contained Thr. Three hemophilic samples were analyzed; two abnormal factor IX molecules contained Ala whereas one contained Thr.

We have reported an immunoassay detecting a polymorphism in plasma coagulation factor IX using a mouse monoclonal antibody (A-1) with allele frequencies of 0.25 for the less frequent allele (95% confidence interval of 0.14 to 0.36) in male donors from New Mexico.[17] A report of a factor IX immunoassay polymorphism using different monoclonal antibodies showed an allele frequency of 0.34 for the less frequent allele (95% confidence interval of 0.17 and 0.52) in 29 Swedish males.[16] In contrast to the A-1 antibody, the monoclonal antibody prepared in Sweden, designated 9.9, partially neutralized factor IXa, whereas the A-1 antibody

Table 3. Heterozygote Detection by Monoclonal Antibody Specific for Thr-148 Allele

			Factor IX Level (U/dL)				
Subject	Sex	Status	IX C	IX·Ag (non, A-1)	IX:Ag (A-1)	X-linked Genotype (Assigned)	*TaqI* Genotype (kb) Polymorphism
II-1	M	Hemophilic	26	62	110	Thr-H	—
II-2	M	Hemophilic	25	—	—	(Thr-H)	—
III-3	F	Obligate	64	72	95	Thr-H/Thr-N	—
III-5	F	Obligate	60	49	18	Thr-H/Ala-N	1.8/1 3
III-5a	M	Normal	108	121	9	Ala-N	—
III-6	F	Obligate	39	42	22	Thr-H/Ala-N	—
III-7	F	Obligate	—	70	16	Thr-H/Ala-N	1.8/1.3
III-7a	M	Normal	120	160	190	Thr-N	1 8
III-8	F	Obligate	72	90	16	Thr-H/Ala-N	—
III-8a	M	Normal	100	118	155	Thr-N	—
III-10	M	Hemophilic	20	29	74	Thr-H	—
IV-2	M	Normal	76	76	100	Thr-N	—
IV-4	M	Hemophilic	15	27	44	Thr-H	—
IV-5	F	Possible	78	110	2	Ala-N/Ala-N	—
IV-6	M	Normal	110	92	1	Ala-N	—
IV-7	M	Normal	78	74	1	Ala-N	—
IV-8	M	Hemophilic	20	27	40	Thr-H	1.8
IV-9	F	Possible	74	85	1	Ala-N/Ala-N	—
IV-10	M	Normal	80	83	1	Ala-N	—
IV-11	M	Normal	80	82	1	Ala-N	—
IV-12	M	Hemophilic	—	44	55	Thr-H	1.8
IV-13	F	Possible	—	69	93	Thr-H/Thr-N	1 8/1.8
IV-14	F	Possible	42	42	64	Thr-H/Thr-N	1.8/1.8
IV-15	F	Possible	45	61	72	Thr-H/Thr-N	—
IV-16	F	Possible	80	80	33	Thr-N/Ala-N	—
IV-17	F	Possible	80	111*	84	Thr-N/Ala-N	—

Factor IX clotting activity (IX:C), antigen levels (IX:Ag) by routine (non, A-1) and specific (A-1 monoclonal) and *TaqI* polymorphic patterns are shown for members of a large pedigree with mild hemophilia B. Clotting and antigen levels are expressed in units per deciliter (U/dL), where normal pooled plasma is assumed to have 100 U/dL. N and H designations for Thr and Ala alleles are for normal and hemophilic genes, respectively. Assay methods for A-1 and non–A-1 assays are explained in the text. All samples were compared to a standard pooled plasma except IV-17.

The female's plasma gave comparable levels of IX:Ag by the two assays. It is probable that she is heterozygous for the Ala allele. Comparison of IX:Ag with a standard plasma from a single male with the Thr allele better defines the heterozygous state in this family member does not inhibit factor IXa clotting activity. (K. Smith, unpublished observations, July, 1986) A further difference between the Swedish antibody and A-1 is that the 9.9 antibody's reactivity with factor IX samples expressing the less frequent allele was 1,000-fold less than polyclonal antibodies or other monoclonal antibodies tested. Although the A-1 antibody has reduced reactivity with factor IX samples expressing Ala, in some cases the apparent antigen is as high as one-fifth of that seen with polyclonal and other monoclonal antibodies. The localization of the epitope to which the Swedish antibody binds and the potential overlap with the sequence to which A-1 binds remains to be established.

The sequence data on the activation peptide confirm the previous report on the activation peptide dimorphism. Three of four individuals showed Thr and their plasma factor IX reacted strongly with A-1, whereas one individual whose activation peptide contained Ala had reduced reactivity in factor IX immunoassays. The possibility that there may be an additional, linked polymorphism in the activation peptide is unlikely since the cDNA sequence of factor IX has been determined by five groups,[14,42-45] and the only difference in these is the codon specifying Thr or Ala at position 148. In addition, recombinant factor IX proteins showed A-1 reactivity as predicted from their known DNA sequence.

The nature of the structural difference between the Thr peptide and the Ala peptide is not known, but there is no apparent difference in clotting activity between the two variants. Because the analysis of A-1 reactivity of hemophilic plasmas could be altered by mutations in regions adjacent to or including the activation peptide, it will be important to test the immunoassay classification for Thr and Ala alleles in hemophilia B samples in which amino acid substitutes are known to be adjacent to the amino terminal region of the activation peptide. Verification of the A-1 classification for hemophilic factor IX could also be obtained by using oligonucleotide probes specific for the DNA polymorphism coding for Thr or Ala in hemophilic DNA.[15]

As this study illustrates, the A-1 immunoassay can establish the carrier state for members of selected families. Analysis of DNA RFLPs is well established for carrier testing, but disadvantages are the time required for isolation and digestion of DNA, the expense of restriction enzymes, and the complexity of analysis of RFLPs when full-length cDNA is used as a probe.

The immunoassay system is useful as a rapid test that provides a valuable reference for the results of DNA analysis in families in which measurable abnormal factor IX is expressed. In addition, linkage data indicate that the heterozygosity for Thr/Ala will predict the utility of DNA analysis when heterozygosity for *Taq*I restriction sites is required. Thus, carriers heterozygous for Thr/Ala are likely to be heterozygous for *Taq*I, and prenatal diagnosis by *Taq*I RFLP analysis should be possible.

The immunoassay may not be useful in some families in which the abnormal gene product is not expressed in sufficient amounts for Thr/Ala classification by immunoassay. In addition, since carrier detection by immunoassay depends on expression of both X chromosome gene products in females, the accuracy of detection will be limited by random inactivation of the X chromosome in hepatocytes, as occurred in borderline values for classification of potential carrier IV-17 in the large family presented. In some families in which the abnormal factor IX gene product is not expressed or present at levels <1 nmol/L, however, the finding of both Thr/Ala alleles expressed in potential carriers can exclude the carrier state when RFLP analyses are not informative.

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Effects of Recombinant α and γ Interferons on the In Vitro Growth of Circulating Hematopoietic Progenitor Cells (CFU-GEMM, CFU-Mk, BFU-E, and CFU-GM) From Patients With Myelofibrosis With Myeloid Metaplasia

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Myelofibrosis with myeloid metaplasia (MMM) is a chronic myeloproliferative disorder due to clonal expansion of a pluripotent hematopoietic progenitor cell with secondary marrow fibrosis. No definitive treatment has as yet been devised for this condition, which shows a marked variability in clinical course. To evaluate whether excessive hematopoietic progenitor cell proliferation could be controlled by recombinant human interferon α (rIFN-α) and γ (rIFN-γ), we studied the effects of these agents on the in vitro growth of pluripotent and lineage-restricted circulating hematopoietic progenitor cells in 18 patients with MMM. A significant increase in the growth (mean ± 1 SEM) per milliliter of peripheral blood of CFU-GEMM (594 ± 253), CFU-Mk (1,033 ± 410), BFU-E (4,799 ± 2,020) and CFU-GM (5,438 ± 2,505) was found in patients as compared with normal controls. Both rIFN-α and rIFN-γ (10 to 10^4 U/mL) produced a significant dose-dependent suppression of CFU-GEMM, CFU-Mk, BFU-E, and CFU-GM growth. Concentrations of rIFN-α and rIFN-γ causing 50% inhibition of colony formation were 37 and 163 U/mL for CFU-GEMM, 16 and 69 U/mL for CFU-Mk, 53 and 146 U/mL for BFU-E, and 36 and 187 U/mL for CFU-GM, respectively. A marked synergistic effect was found between rIFN-α and rIFN-γ: combination of the two agents produced inhibitory effects greater than or equivalent to those of 10- to 100-fold higher concentrations of single agents. These studies (a) confirm that circulating hematopoietic progenitors are markedly increased in MMM, (b) indicate that these presumably abnormal progenitors are normally responsive to rIFNs in vitro, and (c) show that IFNs act in a synergistic manner when used in combination. Because rIFN-γ can downregulate collagen synthesis in vivo, this lymphokine could be particularly useful in the treatment of patients with MMM.

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MYELOFIBROSIS with myeloid metaplasia (MMM) is a chronic myeloproliferative disorder characterized by marrow fibrosis and extramedullary hematopoiesis.[1] There is a marked variability in clinical severity of the disease and in survival, but ~25% of patients die within 18 months of diagnosis.[2-4] Data from studies on patients with a chromosomal marker[5,6] and analysis of glucose-6-phosphate dehydrogenase (G6PD) isoenzymes in a heterozygous woman with MMM[7] suggest the clonal expansion of a multipotent stem cell. Consequently, MMM, like other myeloproliferative syndromes, is not due to excessive proliferation of normal stem cells but is most likely a clonal disease and, by inference, neoplastic in origin.[8]

The clonal expansion of single stem cells in MMM has been strongly supported also by findings of increased numbers of pluripotent (CFU-GEMM, colony-forming unit-granulocyte, erythrocyte, macrophage, megakaryocyte) and lineage-restricted circulating hematopoietic progenitor cells (CFU-Mk, colony-forming unit-megakaryocyte; BFU-E, burst-forming unit-erythroid; CFU-GM, colony-forming unit-granulocyte–macrophage).[9-18] In contrast, G6PD data[7] and studies on the origin of human bone marrow fibroblasts[19] and marrow-derived fibroblast colonies[20] suggest that myelofibrosis is a secondary or reactive process, possibly mediated by platelet-derived growth factor (PDGF) and platelet factor 4.[21-24]

Interferons (IFNs) are a group of naturally occurring inducible proteins that exert several biologic actions[25,26] including, at least in vitro, modulation of hematopoiesis.[27,28] IFNs have been shown to have inhibitory effects on the growth of hematopoietic progenitor cells from normal marrow[29-31] as well as from patients with chronic myeloid leukemia.[32]

Due to these properties, IFNs may be considered potentially useful for controlling the clonal expansion of hematopoietic progenitors and the clinical manifestations of MMM. The aim of this study was to analyze the effects of rIFN-α and rIFN-γ on the in vitro growth of pluripotent (CFU-GEMM) and lineage-restricted (CFU-Mk, BFU-E, CFU-GM) hematopoietic progenitor cells from patients with MMM.

MATERIALS AND METHODS

Patients. Eighteen patients with myelofibrosis with myeloid metaplasia were studied. The clinical data regarding these patients are given in Table 1. The diagnosis was based on the following criteria[4]: splenomegaly, leukoerythroblastic blood picture with RBC anisopoikilocytosis and characteristic teardrop cells, increased reticulin, and at least focal fibrosis in the bone marrow trephine biopsy. None of the patients had a clinical picture of malignant myelosclerosis, a history of chronic myeloid leukemia, or other identifiable explanation for the marrow fibrosis. No patient had received chemotherapy before the study. Androgens and/or corticosteroids were discontinued at least 2 weeks before cultures were performed. Ten (5 men, 5 women) hematologically normal volunteers aged 20 to 60 years were chosen as controls and studied in parallel with the patients.

Cell separation procedures. Peripheral blood cells were obtained from patients and controls, who had given informed

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Table 1. Clinical Data of Patients at Time of Study

Case	Age/Sex	Time from Diagnosis (mo)	Splenectomy	Hb g/dL	WBC 10^9/L	Platelets 10^9/L	Marrow Cellularity
1	55/M	75	—	9.9	23.5	55	Increased
2	33/F	128	—	12.7	77.8	335	Decreased
3	64/M	192	Yes	15.3	6.7	528	Increased
4	68/M	153	Yes	12.6	24.7	1,122	Increased
5	54/M	72	—	15.8	14.4	552	Normal
6	70/F	25	—	10.7	67.8	122	Increased
7	62/F	19	—	12.4	4.9	263	Normal
8	73/F	10	Yes	7.7	17.8	266	Normal
9	45/F	108	Yes	10.3	95.0	761	Decreased
10	45/F	24	—	12.0	13.0	415	Decreased
11	64/F	3	—	7.0	25.0	361	Decreased
12	57/M	63	—	10.3	13.2	225	Normal
13	62/M	84	—	9.6	3.6	121	Normal
14	65/M	18	Yes	12.2	53.2	196	Increased
15	58/M	61	—	10.9	3.2	148	Normal
16	55/M	60	—	9.8	6.0	143	Decreased
17	77/M	36	—	10.3	5.0	103	Normal
18	78/F	72	—	8.0	26.6	90	Normal

consent. Light-density mononuclear cells (LDMNCs) were separated by centrifugation on a Ficoll-Hypaque gradient (density 1.077 g/mL) at 400 g for 40 minutes at 20°C. Interface cells were washed three times in RPMI 1640 medium (GIBCO, Grand Island, NY) and suspended in Iscove's modified Dulbecco's medium (IMDM, Seromed, Berlin).

In vitro assays. The assay for GFU-GEMM, CFU-Mk, BFU-E, and CFU-GM was carried out as described in detail elsewhere.[33,34] In briefly, 1×10^5 LDMNCs for patients with leukocyte counts $>20 \times 10^6$/mL, 5×10^5 LDMNCs for patients with $<20 \times 10^6$/mL WBCs, and 1×10^6 LDMNCs for controls were plated in 35-mm Lux Petri dishes (Miles Laboratories, Naperville, IL) with and without different concentrations (10 to 10^4 U/mL) of rIFN-α and rIFN-γ. In preliminary experiments, erythropoietin and medium conditioned by peripheral blood leukocytes in the presence of 1% phytohemagglutinin (PHA-LMC) requirements for the optimal formation of CFU-GEMM and CFU-Mk were studied. Therefore, LDMNCs were seeded in 1-mL aliquots of IMDM containing 30% human fresh frozen plasma from a single donor; 5% medium conditioned by peripheral blood leukocytes[35] in the presence of 1% PHA (PHA-HA 15, Burroughs Wellcome, Research Triangle Park, NC); 5×10^{-5} mol/L 2-mercaptoethanol (Sigma, St Louis); 1.0 U erythropoietin (Step III, Connaught Laboratories, Toronto), and 0.9% (wt/vol) methylcellulose.

After incubation for 14 days at 37°C in a humidified atmosphere supplemented with 5% CO_2, the cultures were examined with an inverted microscope. Four dishes were set up for each individual data point. Mixed colonies (CFU-GEMM) defined as containing at least erythroid and granulocytic cells by their in situ appearance, megakaryocytic colonies (CFU-Mk) defined as containing more than eight cells of varying size and characterized by a translucent hyaline cytoplasm and highly refractile cytoplasmic border, erythroid bursts (BFU-E) with >500 cells, and granulocyte-macrophage colonies (CFU-GM) with >40 cells were all scored from the same plates. To confirm their megakaryocytic nature, a representative number of CFU-Mk were picked up and immunologically analyzed[36] with the monoclonal antibody C 17.28 against platelet glycoprotein IIIa[37] (kindly provided by Dr P. Lansdorp).

The total number of LDMNCs obtained per unit volume of peripheral blood after separation on a Ficoll-Hypaque gradient was calculated to serve as a basis for the determination of the number of colonies grown per unit volume of peripheral blood. The following formula was used: Hematopoietic progenitors/mL = colonies/dish × LDMNCs/mL blood/plated LDMNCs (no.). Although such a calculation may be erroneous due to a possible variability in the recovery of hematopoietic progenitors from LDMNCs, it provides the only means of comparing progenitor numbers in patients with highly different WBC counts. The reduction of colony numbers due to rIFNs was expressed as percentage of inhibition of growth (mean ± 1 SEM) as compared with the number of hematopoietic progenitor cells grown per 1 to 5×10^5 or 1×10^6 plated cells.

In preliminary experiments, cell dose–response curves were set up for both patients and controls, and a linear relationship between the number of hematopoietic progenitor cells and the number of LDMNCs plated was observed. Several batches of PHA-LCM were used in this study; however, these were always produced by peripheral blood mononuclear cells from the same individual and tested for comparable stimulative properties before use. Similarly, a single donor was used to provide human plasma throughout the whole study.

Recombinant IFNs and monoclonal antibodies against IFNs. rIFN-α (specific activity: 3.3×10^8 U/mg protein) was kindly provided by Karl Thomae, Biberach, FRG; rIFN-γ (specific activity: 2.4×10^7 U/mg protein) was produced by Biogen Research, Cambridge, MA, and kindly furnished by Bioferon, Laupheim, FRG.

The monoclonal antibodies EB-1 against rIFN-α, GZ-4 and C-15 against rIFN-γ, also supplied by Karl Thomae, were used as a 1:200 dilution of mouse ascites. To neutralize the activity of rIFN-α and rIFN-γ, monoclonal antibodies were incubated with rIFN preparations for 90 minutes at room temperature before culture.

Statistical analysis. Statistical analyses were performed with the statistical packages Statfast (Statsoft, Tulsa, OK) and Statview (BrainPower, Calabasas, CA) run on a MacIntosh Plus personal computer (Apple Computer, Cupertino, CA). Four plates were scored for each data point per experiment and the results were expressed as the mean ± 1 SEM. IFN concentrations resulting in 50% inhibition of colony formation were derived by regression analysis.

RESULTS

Circulating CFU-GEMM, CFU-Mk, BFU-E, and CFU-GM in patients with MMM. In preliminary experiments, cell dose–response curves were plotted. Linearity could be observed in a range from 2.5 × 10^5 to 1 × 10^6 plated cells with normal subjects, and from 1 × 10^5 to 5 × 10^5 plated cells with MMM patients.

Numbers of pluripotent and lineage-restricted progenitor cells grown per unit volume from the blood of the 18 patients with MMM are shown in Fig 1. Baseline growth (mean ± SEM) was significantly increased for CFU-GEMM (594 ± 253 *v* 32 ± 3, $P \leq .05$), CFU-Mk (1,033 ± 410 *v* 53 ± 6, $P \leq .005$), BFU-E (4,799 ± 2,020 *v* 267 ± 43, $P \leq .05$), and CFU-GM (5,438 ± 2,505 *v* 251 ± 53, $P \leq .05$) when compared with the growth of controls. In 3 of 18 patients, mixed colonies could not be detected, whereas megakaryocytic colonies were observed in all samples studied. Colony growth was within normal range in two patients for CFU-GEMM and CFU-Mk and in four patients for BFU-E and CFU-GM. No particular differences in progenitor cell number were observed between patients who had undergone splenectomy and those who had not.

Effects of rIFN-α and rIFN-γ on growth of circulating CFU-GEMM, CFU-Mk, BFU-E, and CFU-GM. The effects of rIFNs on colony formation were studied in 17 patients. Due to baseline colony growth, the action of rIFNs was only evaluable in 13 cases for CFU-GEMM and in 16 for CFU-Mk and BFU-E, whereas it could be measured in all cases for CFU-GM growth.

When peripheral blood mononuclear cells (LDMNCs) were exposed throughout the entire culture period to rIFN-α and rIFN-γ, a statistically significant, dose-dependent suppression of colony growth from CFU-GEMM, CFU-Mk, BFU-E, and CFU-GM was observed (Fig 2). Regression analysis showed that inhibition was exponentially related (linearly in a semilog plot) to rIFN concentration over the range tested (10 to 10^4 U/mL). The rIFN-α and rIFN-γ concentrations causing 50% inhibition of colony growth were 37 and 163 U/mL for CFU-GEMM, 16 and 69 U/mL for CFU-Mk, 53 and 146 U/mL for BFU-E, and 36 and 187 U/mL for CFU-GM, respectively. Individual patients exhibited variable sensitivity to the inhibitory effects of rIFN-α and rIFN-γ, although no one failed to respond in some degree to these agents. For example, in patient 1, both rIFNs caused a reduction in CFU-GM growth <50% at any of the concentrations tested (10 to 10^4 U/mL); in patient 8, rIFN-γ reduced BFU-E growth by only 49% even at 10^4 U/mL.

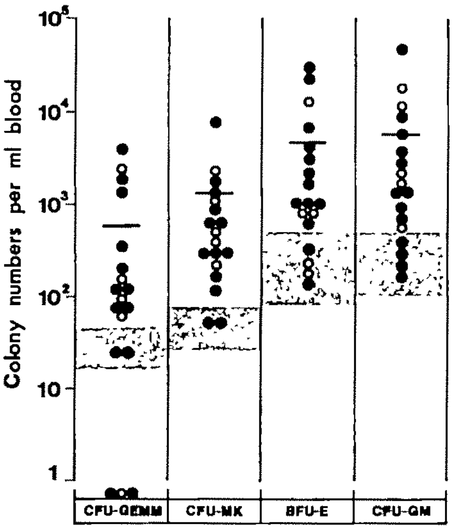

Fig 1. Hematopoietic progenitor CFU-GEMM, CFU-Mk, BFU-E, and CFU-GM growth obtained from peripheral blood mononuclear cells in the 18 patients studied. Each point represents the mean of four separate dishes. Not splenectomized (●); splenectomized (○). Shaded areas represent the ranges of growth in the controls (n = 10).

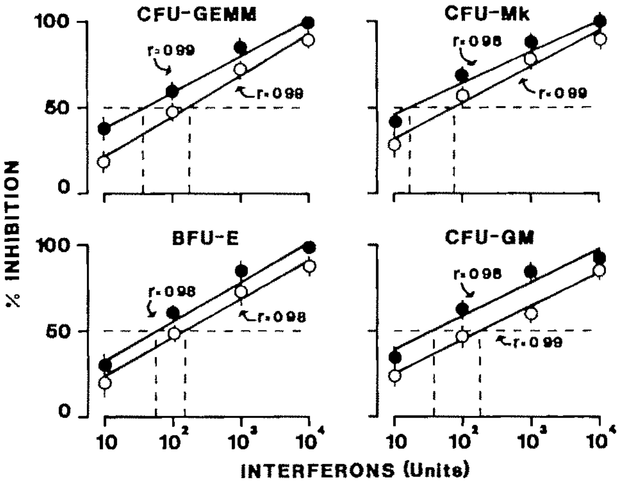

Fig 2. Effect of rIFN-α (●——●) and rIFN-γ (○——○) on the growth of pluripotent (CFU-GEMM), megakaryocytic (CFU-Mk), erythroid (BFU-E), and granulocyte-macrophage (CFU-GM) progenitor cells from peripheral blood mononuclear cells of 17 patients with myelofibrosis with myeloid metaplasia. Each point represents the percentage of inhibition expressed as the mean ± SEM. When compared with that of control cultures, the inhibitory effect of rIFN-α was statistically significant at a dose of 10 U/mL on CFU-GEMM ($P < .0005$), CFU-Mk ($P < .0005$), BFU-E ($P < .0005$), and CFU-GM ($P < .0005$); the inhibitory effect of rIFN-γ was statistically significant at the dose of 10 U/mL on CFU-GEMM ($P < .005$), CFU-Mk ($P < .0005$), BFU-E ($P < .01$), and CFU-GM ($P < .0005$). Regression lines for each rIFN were derived, and concentrations of rIFN resulting in 50% inhibition of colony formation were calculated from these lines.

In three normal subjects (data not shown), a dose-dependent suppression of colony growth was also observed, exponentially related to rIFN concentration over the tested range (10 to 10^4 U/mL). Dose–response curves were not significantly different from those observed in patients with MMM.

Effects of combined doses of rIFN-α and rIFN-γ on growth of circulating CFU-GEMM, CFU-Mk, BFU-E, and CFU-GM. Table 2 reports experiments in three normal subjects and five patients with MMM, in whom LDMNCs were exposed throughout the entire culture period to rIFN-α and rIFN-γ used alone (10 to 10^3 U/mL) or in combination (10 to 10^2 U/mL). Considering patients with MMM first, rIFN-α and rIFN-γ used in combination at the dose of 10 U/mL exerted a marked synergism in suppressing hematopoietic progenitor cell growth. The percentages of inhibition (mean ± 1 SEM) observed using 10 U/mL of each rIFN in combination were significantly higher than those obtained with 10^2 U/mL of rIFN-α or rIFN-γ alone and not signifi-

Table 2. Percentages of Growth Inhibition (mean ± SEM) Obtained in Three Normal Subjects and Five Patients With Myelofibrosis With Myeloid Metaplasia Using rIFN-α and rIFN-γ Alone or in Combination

rIFNS (U/mL)	CFU-GEMM	CFU-Mk	BFU-E	CFU-GM
	(Percent) Inhibition in Colony Growth (%) (Mean ± 1 SE)			
Normal subjects (3)				
α (10)	34 ± 4	45 ± 12	35 ± 3	39 ± 5
γ (10)	32 ± 6	35 ± 9	16 ± 6	34 ± 6
$\alpha + \gamma$ (10 + 10)	91 ± 3*	87 ± 5*	88 ± 4*	72 ± 6
α (10^2)	62 ± 2	69 ± 3	58 ± 3	66 ± 16
γ (10^2)	46 ± 6	55 ± 3	37 ± 6	47 ± 14
$\alpha + \gamma$ ($10^2 + 10^2$)	98 ± 1	97 ± 2	99 ± 1	95 ± 1
α (10^3)	86 ± 5	90 ± 3	90 ± 4	74 ± 17
γ (10^3)	70 ± 4	79 ± 4	53 ± 3	58 ± 14
Patients with MMM (5)				
α (10)	36 ± 12	44 ± 10	28 ± 6	30 ± 6
γ (10)	6 ± 3	26 ± 7	9 ± 3	16 ± 6
$\alpha + \gamma$ (10 + 10)	84 ± 7*	87 ± 6	79 ± 5*	72 ± 13*
α (10^2)	54 ± 10	74 ± 9	57 ± 7	59 ± 13
γ (10^2)	31 ± 9	55 ± 9	43 ± 6	35 ± 16
$\alpha + \gamma$ ($10^2 + 10^2$)	91 ± 9†	97 ± 1†	92 ± 6†	85 ± 8†
α (10^3)	82 ± 2	91 ± 4	88 ± 4	87 ± 4
γ (10^3)	72 ± 7	80 ± 9	76 ± 13	65 ± 16

*Significantly ($P < .05$) higher than the value obtained with 10^2 U/mL rIFN-α or rIFN-γ.

†Not significantly different ($P \geq .05$) from the value obtained with 10^3 U/mL rIFN-α or rIFN-γ.

cantly different from those obtained using 10^3 U/mL of rIFN-α or rIFN-γ alone.

Results obtained in three normal subjects with combinations of IFNs showed that the percentages of growth inhibition were comparable to those obtained from patients with MMM (Table 2).

Abrogation of the inhibitory activity of IFNs by MoAbs anti-IFN. Experiments carried out in three normal subjects and three patients with MMM clearly showed that the inhibitory effects of rIFNs were completely removed using monoclonal antibodies to neutralize rIFN-α or rIFN-γ selectively. Data concerning CFU-GM were as follows. rIFN-α at 10^3 U/mL significantly inhibited colony formation in three normal subjects (mean inhibition ± 1 SD: 71% ± 5%) and three MMM patients (79% ± 6%), but these inhibitory effects were removed by preincubation of IFN-α with the monoclonal antibody EB-1 (4% ± 9%, and −5% ± 8%, respectively). The inhibitory effects of rIFN-γ at 10^3 U/mL (58% ± 8% in normal subjects and 64% ± 17% in patients with MMM) were similarly prevented by preincubation with the specific monoclonal antibody (−3% ± 8% and −4% ± 10%, respectively). The same was observed in experiments on IFN synergism: inhibition of CFU-GM in normal subjects (69% ± 9%) and patients with MMM (72% ± 8%) was abolished by preincubation of IFNs with monoclonal antibodies (2% ± 8%, and 9% ± 5%, respectively). Similar findings were obtained with the other hematopoietic progenitors.

DISCUSSION

Consistent with previous reports,[9-17] the in vitro growth of hematopoietic progenitor cells CFU-GEMM, CFU-Mk, BFU-E, and CFU-GM was homogeneously increased ~20-fold in our MMM patients as compared with normal controls. The enlargement of mixed and megakaryocytic progenitor cell compartments is in keeping with the hypothesis that MMM is due to a primary defect at the level of the pluripotent hematopoietic stem cell, which confers on it a proliferative advantage over its normal counterparts.[8]

With regard to the effects of interferons, our data clearly indicate that the growth of circulating hematopoietic progenitors from patients with MMM was suppressed in a dose-dependent manner by rIFNs. The absence of chromosomal markers in this group of patients did not allow us to investigate whether the target of IFN activity was the normal or the neoplastic population. Studies on individuals with myeloproliferative syndromes who were double heterozygous for G6PD, however, have indicated that clonal progenitors are usually >90%.[6] By inference, inhibitions >50% would indicate inhibition of the abnormal population. The observation that the responsiveness of hematopoietic progenitors from MMM patients to rIFNs was equivalent to the responsiveness of normal progenitor cells implies that the presumably neoplastic progenitors of patients with MMM maintain physiologic properties. This concept is clearly supported by the fact that such progenitors are capable of differentiating into circulating blood cells, although with variable efficiency.

Data reported in Table 2 clearly indicate that IFNs act in a synergistic manner when used in combination. Synergism between IFNs in the inhibition of normal bone marrow-derived CFU-GEMM, BFU-E, and CFU-GM growth has been reported.[38,39] Although this may be consistent with the existence of different cell surface receptors for IFN-α and IFN-γ,[40] synergy between IFNs is more likely mediated through auxiliary effector cells activated by IFN-γ.[39] In this study, we found a marked synergism between rIFN-α and rIFN-γ at doses as low as 10 U/mL in the inhibition of peripheral blood CFU-GEMM, CFU-Mk, BFU-E, and CFU-GM growth in patients with MMM. This suppressive effect most probably was due to the rIFNs themselves because it was obtained with highly purified rIFN preparations and the suppression was selectively abolished by monoclonal antibodies against rIFN-α and rIFN-γ. It is well known that rIFNs, especially when used at high doses, have many side effects in vivo.[41] Therefore, for this reason also, the synergistic suppressive activity on hematopoietic progenitor cell growth has potential relevance for the use of IFNs in vivo.

The aim of this work was to investigate the effects of IFNs under optimal conditions of culture. An important question is whether the inhibitory effects of IFNs on hematopoietic progenitors can be reversed by increasing the concentrations of growth factors. Raefsky et al[39] found that increasing amounts of colony-stimulating activity could partially block IFN inhibition of human CFU-GM. Conversely, Mamus et al[42] showed that the inhibitory action of rIFN-γ on normal human BFU-E and CFU-E was not prevented or reversed by erythropoietin. Clearly, further studies based on the use of recombinant growth factors and IFNs are needed to clarify these important interactions between negative and positive molecules, which might be relevant for clinical applications.

A major question is whether the present in vitro findings on the inhibitory effects of IFNs may represent a basis for future clinical trials. At present, treatment of patients with MMM is largely symptomatic, ie, directed toward specific manifestations such as anemia, infections, bleeding, portal hypertension, hypersplenism, or splenic infarction. No treatment has had a clear beneficial effect on the basic pathogenetic process. Thus, future clinical trials aimed at investigating the use of rIFNs in the treatment of MMM may be based on two rationales. First, IFNs could be used in subsets with poorer prognosis of such patients to prevent death by complications such as infections, bleeding, or blast crisis.[3,4] Although normal progenitors are inhibited by IFNs to the same degree as abnormal progenitors, suppression of the clonal expansion of a pluripotent hematopoietic progenitor cell might result in restoration of normal hematopoiesis. Second, treatment with IFNs could represent an approach to reduce hematopoietic marrow hyperproliferation and reverse the pathologic process of myelofibrosis, osteosclerosis, and extramedullary hematopoiesis in patients diagnosed early. From this point of view, interesting findings have recently been reported by Granstein et al.[43] These investigators have shown in a mouse model that rIFN-γ can downregulate collagen synthesis in vivo, and have suggested the possibility that this lymphokine may be useful in the treatment of disease states characterized by excessive fibrosis. In humans, trials with recombinant IFNs have already been carried out in chronic myeloproliferative disorders; encouraging results, including disappearance of the Philadelphia chromosome in some cases, have been obtained in chronic myelogenous leukemia.[44,45] Due to the high sensitivity of MMM progenitors to rINFs and based on preliminary results from in vivo studies, we can anticipate that clinical trials should initially use very low doses of these drugs.

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B-Cell Growth Factor Receptor Expression and B-Cell Growth Factor Response of Leukemic B Cell Precursors and B Lineage Lymphoid Progenitor Cells

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The purpose of this study was to analyze the expression of B cell growth factor (BCGF) receptors and to elucidate the biologic effects of biochemically purified natural BCGF at the B cell precursor stage of human B lineage lymphoid differentiation. The specific binding of radioiodinated high-mol-wt BCGF (^{125}I-HMW-BCGF) and low-molecular-wt BCGF (^{125}I-LMW-BCGF) to fresh marrow blasts from B cell precursor acute lymphoblastic leukemia (ALL) patients was initially investigated. The estimated number of radioiodinated BCGF molecules bound per blast ranged from undetectable to 24.3×10^3 for HMW-BCGF, and from 11.5×10^3 to 457.8×10^3 for LMW-BCGF. In ^{3}H-TdR incorporation assays, 75% of cases showed a significant response to LMW-BCGF with a median stimulation index of 9.3. By comparison, only 33% of cases showed a significant response to HMW-BCGF with a median stimulation index of 2.4. Subsequently, B cell precursor colony assays were performed to assess and compare the biologic effects of BCGF on leukemic B lineage lymphoid progenitor cells. Among 28 cases studied, 57% responded to both HMW-BCGF and LMW-BCGF, 21% responded only to LMW-BCGF, and the remaining cases showed no proliferative response to either growth factor. The response patterns of virtually pure populations of FACS-sorted leukemic B cell precursors were essentially identical to the proliferative responses of unsorted leukemic B-cell precursors. Synergistic effects between HMW-BCGF and LMW-BCGF were observed in 80% of the cases that responded to both. The numbers of cell-bound radioiodinated BCGF molecules, the stimulation indices, as well as the number of B cell precursor colonies in BCGF-stimulated cultures showed a marked interpatient variation. Patients with structural chromosomal abnormalities (SCAs) involving 12p11-13 or patients with a Philadelphia chromosome showed a greater HMW-BCGF response at the level of leukemic progenitor cells than did other patients ($P = .02$). The LMW-BCGF response was significantly greater for patients with SCA than for patients without SCA ($P = .04$). The response of leukemic progenitor cells to HMW-BCGF or LMW-BCGF did not correlate with sex, age, disease status, FAB morphology, WBC at diagnosis, or immunophenotype. To our knowledge, this study represents the first detailed analyses of BCGF receptor expression and BCGF effects in B cell precursor ALL. The data presented provide direct evidence for the expression of functional receptors for both HMW-BCGF and LMW-BCGF in B cell precursor ALL.

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HEMATOPOIETIC growth factors (GFs) are regulatory polypeptides/glycoproteins that influence proliferation as well as differentiation of hematopoietic cells.[1] The availability of purified natural and cloned GF proteins, which has made detailed analyses of their biologic effects and mechanisms of action possible, represents an important step in the elucidation of the complexities involved in regulation of normal v leukemic hematopoiesis. Several GFs have been described that influence proliferation, activation, or differentiation of human B lymphocytes.[2-36] These GFs include B cell growth factors (BCGFs),[8-17,34-36] B cell differentiation factor (BCDF),[13,16,17] B cell activation factor (BAF),[19] pre-B cell growth factor,[20] B cell inhibitory factor (BIF),[18] interleukin 2 (IL 2),[4,5,21-27] γ interferon (γ-IFN),[22,27,28] and interleukin 1 (IL 1).[29-33] BCGFs have been derived from normal B and T lymphocytes, B lineage and T lineage lymphoma cell lines, T-T hybridomas, T cell clones, and Epstein-Barr virus (EBV) transformed B cells.[6-10] Human BCGFs are heterogeneous with high-mol-wt and low-mol-wt forms.[6,9,12] A 60,000-dalton, high-mol-wt BCGF (HMW-BCGF) has been biochemically purified from the supernatants of T-ALL, a human T cell line[12]; and a 12,000-dalton, low-mol-wt BCGF (LMW-BCGF) has been purified from lectin-stimulated peripheral blood mononuclear cell-conditioned medium.[9] In previous studies, we showed that both HMW-BCGF and LMW-BCGF in purified form enhance the proliferative activity of preactivated human B lymphocytes.[9,12] Specific receptors for HMW-BCGH were demonstrated on activated mature human B lymphocytes using ^{125}I-HMW-BCGF as a probe.[12] Similarly, receptor binding studies and Scatchard analysis using ^{125}I-LMW-BCGF revealed both high-affinity and low-affinity receptor sites for LMW-BCGF on freshly prepared, anti-μ activated peripheral blood B lymphocytes as well as on BCGF-dependent human B cell lines.[37] Besides activated mature B lymphocytes, some B lineage non-Hodgkin's lymphoma cells[38] and hairy cell leukemia cells,[39] representing relatively mature stages of differentiation within the B precursor pathway, have been shown to respond to BCGF. The stage in

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human B cell ontogeny during which B lineage cells express BCGF receptors and become responsive to BCGF, however, is as yet undefined.

The purpose of the present study was to analyze the expression of surface BCGF receptors and to elucidate the growth regulatory effects of BCGF at the B cell precursor stage of human B lineage lymphoid differentiation. We investigated the specific binding of ^{125}I-HMW-BCGF and ^{125}I-LMW-BCGF to BCGF receptors on freshly obtained leukemic marrow B cell precursors from 28 B cell precursor ALL patients. BCGF-induced stimulation of DNA synthesis as well as BCGF-induced promotion of B cell precursor colony formation were determined to evaluate the functional integrity of detected BCGF receptors. Using FACS-sorted, virtually pure populations of fresh leukemic marrow blasts and B cell precursor colony assays, we then studied the biologic effects of HMW-BCGF and LMW-BCGF as well as their combined effects at the level of leukemic B lineage lymphoid progenitor cells. We also examined how the BCGF response of leukemic progenitor cells correlates with more commonly measured disease- and host-related diagnostic parameters. To our knowledge, this report is the first detailed comparative analysis of BCGF receptor expression at very early stages of B lineage lymphoid differentiation. We provide evidence for (a) expression of functional BCGF receptors on leukemic B cell precursors and their progenitor blasts, and (b) a relationship between responsiveness to BCGF and structural chromosomal abnormalities.

MATERIALS AND METHODS

Patient material. 28 patients with B cell precursor ALL were included in this study. Bone marrow aspirate sample were procured by routine procedures. Morphological classification was performed on Wright's-Giemsa–stained slides according to a modification of the original French-American-British (FAB) nomenclature.[40] Diagnosis was based on morphological, cytochemical, and surface marker profiles of marrow blasts.

Immunologic surface marker analyses Marrow blasts were isolated by a single centrifugation of aspirate samples on Ficoll-Hypaque (1.077 g/cm^3) Immunologic marker analyses by indirect immunofluorescence (IF) and flow cytometry were performed as previously described[41,42] using monoclonal antibodies (mAb) BA-1 (CD24), BA-2 (CD9), BA-3 (CD10/common ALL antigen, CALLA), and B43(CD19) which define B lineage associated surface determinants; B43, which defines a B lineage restricted surface determinant; as well as T cell panel mAb 35.1 (CD2), T101 (CD5), and 3A-1 (CD7), which recognize T lineage differentiation antigens. FITC-labeled goat F(ab)'$_2$ anti-mouse IgG (Cappel Laboratories, Cochranville, NC) served as secondary antibody. Cell surface immunglobulin (sIg) was assayed by direct IF with FITC-conjugated goat anti-human total Ig (Kallestad, Austin, TX). An IgG2a murine myeloma protein (UPC 10; Litton Bionetics, Charleston, SC) substituted for mAb was used for background fluorescence. Cells were analyzed for IF using a Spectrum III Cytofluorograph (Ortho Diagnostics, Raritan, NJ). Samples were considered positive when >20% of cells bound the antibody used.

Cytogenetic analysis of bone marrow blasts Banded chromosome analyses were performed on direct, overnight, and in some cases, synchronized preparations of fresh bone marrow samples. Several methods of banding were used, including G-banding with Wright's stain, GTP-banding, and QFQ-banding. Chromosome abnormalities were designated using the International System for Human Cytogenetics 1985 Nomenclature[43] An abnormal clone was defined as >2 metaphase cells with identical structural abnormalities or identical extra chromosomes, or >3 metaphases with identical missing chromosomes For the case to be considered cytogenetically normal, ie, normal diploid, at least 15 metaphase cells had to be examined and all found normal. The details of the methodology have been published elsewhere.[44-46]

Human BCGFs. We used highly purified preparations of human HMW-BCGF and LMW-BCGF for radioiodination and BCGF receptor expression studies. Biologically active HMW-BCGF was prepared from supernatants of a phytohemagglutinin (PHA)-stimulated T-ALL cell line and purified to biochemical homogeneity using immunoabsorption (×2) and high-performance liquid chromatography (HPLC)[12] The final protein eluted as a sharp peak at 60,000 mol wt. The specific biologic activity of purified HMW-BCGF, as evaluated in B cell stimulation assays[11,12,14,47,48] and expressed in half-maximal stimulation units, was estimated as 5×10^5 U/mg pure protein. This HMW-BCGF was radiolabeled using the Bolton-Hunter reagent as reported.[12] The radiolabeled, functionally active protein showed only a single band at 60,000 mol wt when analyzed by sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography, as previously reported[12] ^{125}I-HMW-BCGF had a specific activity of 0.8×10^{18} cpm/mol. Biologically active LMW-BCGF was prepared from a pooled PHA-stimulated, peripheral blood mononuclear cell-conditioned medium and purified to biochemical homogeneity as previously described[9] using a series of column chromatograpic steps including preparative scale diethylaminoethanol (DEAE) chromatography, hydroxyapatite column chromatography, ion-exchange, and size exclusion HPLC. The final protein eluted as sharp peak at 12,000 mol wt and produced a single band when analyzed by SDS-PAGE and silver staining, as previously reported.[9] The specific biologic activity of LMW-BCGF was estimated to be 10^6 U/mg pure protein by BioRad assays. LMW-BCGF was radioiodinated by a solid-phase iodination technique using Iodo-gen (Pierce Chemical, Rockford, IL) as previously described[37] and retained ~50% of its biologic activity as determined by a probit analysis.[37] The specific activity of ^{125}I-LMW-BCGF was 0.8×10^{17} cpm/mol. ^{125}I-HMW-BCGF and ^{125}I-LMW-BCGF were used to analyze BCGF receptor expression in B cell precursor ALL as described hereinafter. Because of (a) the need for large quantities of stable HMW-BCGF and LMW-BCGF to perform comparative and detailed biologic assays on fresh marrow cells from a large number of B cell precursor ALL patients over several months, (b) the relatively low yield of our biochemical purification procedures, and (c) the relative instability of homogeneous BCGF preparations (highly purified BCGFs remain biologically active only 2 to 4 weeks), we chose to use partially purified HMW-BCGF (ie, material obtained from PHA-stimulated T-ALL supernatants by affinity chromatography and repeated steps of immunoabsorption against BCGF/1/C2 mAb directed against HMW-BCGF but not reactive with LMW-BCGF, which was coupled to Sepharose) and partially purified LMW-BCGF (ie, material obtained from crude PHA-conditioned media by DEAE chromatography followed by hydroxylapatite column chromatography) in our BCGF response studies Partially purified BCGF preparations did not contain detectable amounts of IL 1, IL 2, BPA/IL 3, GM-CSF, G-CSF, or γ-IFN as determined by previously described standard bioassays[11,12,14,27,28,47,49-54] but were able to induce proliferation of activated normal B cells with a maximal stimulation index of 16.9 for HMW-BCGF and 23.6 for LMW-BCGF. This is consistent with our previous reports[9,12] The actual BCGF protein concentrations in partially purified BCGF preparations were estimated by comparing their biologic activity on preactivated normal B cells side by side with the biologic activity of known concentrations of highly purified, fresh BCGF preparations. Specifically, 10% (vol/vol) partially purified HMW-BCGF and 2 ng/mL highly purified HMW-BCGF both had 1U (half-maximal stimulation unit)/mL BCGF activity, as determined in the SAC assay,[48] and 5% (vol/vol) partially purified LMW-BCGF and 1 ng/mL highly purified LMW-BCGF both had 1U (half-maximal stimulation unit)/mL BCGF activity, as determined in the anti-μ B cell stimulation assay[11,12,14,47] (data not shown).

BCGF receptor binding and receptor function studies. Radiolabeled and biologically active BCGF preparations were obtained as described above. The specific binding of ^{125}I-HMW-BCGF and ^{125}I-LMW-BCGF to fresh leukemic marrow B cell precursor blasts was evaluated as previously described in detail.[12,37] In brief, leukemic B cell precursor blasts were washed several times in cold RPMI 1640 and were resuspended at 1×10^5 cells/100 μL ^{125}I-HMW-BCGF (50,000 cpm) or ^{125}I-LMW-BCGF (50,000 cpm) in RPMI and incubated for 1 hour at 4°C. At the end of incubation, cells were washed extensively and cell-associated radioactivity was quantitated using a γ 7000 scintillation counter (Beckman Instruments, Irvine, CA). For cold competition, unlabeled highly purified HMW-BCGFs or LMW-BCGFs were added to the samples simultaneously with the radioiodinated HMW-BCGF or LMW-BCGF. The specific binding was calculated in the presence of tenfold excess cold BCGF as reported.[12,37] Data were expressed as (a) percentage of inhibitable binding, (b) amount of cell-associated cpm/10^5 cells inhibitable by excess unlabeled BCGF, and (c) the number of estimated BCGF molecules specifically bound per cell. ^{125}I-HMW-BCGF had a specific activity of 0.8×10^{18} cpm/mol. Hence, 1 cpm associated with 10^5 cells and inhibitable by excess cold HMW-BCGF corresponded to 7.5 molecules of radioiodinated HMW-BCGF specifically bound to each cell [6×10^{23} molecules (≈1 mol) times 1 cpm divided by (0.8×10^{18} cpm $\times 10^5$ cells)] ^{125}I-LMW-BCGF had a tenfold less specific activity (≈0.8×10^{17} cpm/mol). Therefore, each inhibitable cpm bound to 10^5 cells corresponded to 75 molecules of radioiodinated LMW-BCGF molecules specifically bound to each cell. The calculated number of BCGF molecules bound per cell may not be equivalent to the actual number of BCGF receptors per cell, since the data were obtained from binding assays with a single concentration of ligand and not from Scatchard analyses. The functional integrity of detected BCGF receptors were analyzed in standard ^{3}H-TdR incorporation assays using partially purified BCGF (5 ng/mL HMW-BCGF or 2 ng/mL LMW-BCGF) as described.[11,12,14,47] Initial experiments using fresh leukemic blasts from three B cell precursor ALL patients showed a dose-dependent increase in ^{3}H-thymidine incorporation in the presence of BCGF with maximum stimulation obtained at 5 ng/mL HMW-BCGF or 2 ng/mL LMW-BCGF (data not presented). Therefore, subsequent experiments were performed using 5 ng/mL HMW-BCGF or 2 ng/mL LMW-BCGF. Data were expressed as stimulation indices, which were calculated as the ratios of the amounts of ^{3}H-TdR incorporation by BCGF-stimulated blasts to the amounts of ^{3}H-TdR incorporation by unstimulated blasts (background controls). In parallel experiments, cells were cultured for 72 hours with HMW-BCGF or LMW-BCGF, and subsequently their immunophenotypes were analyzed on cytospin slides to exclude BCGF-induced proliferation of sIg$^+$ normal mature B cells as an explanation of increased ^{3}H-TdR incorporation in BCGF-stimulated cultures.

FACS FACS was used to isolate virtually pure populations of leukemic B cell precursors In brief, following density gradient separation on Ficoll-Hypaque and depletion of adherent cells by adherence to plastic surfaces, highly blast-enriched, freshly obtained bone marrow mononuclear cells were put into a cold mixture of 10 μg/mL affinity-purified B43 (anti-CD19) and 10 μg/mL affinity-purified 24.1 (anti-CD10/CALLA) mAb at 1×10^7 cells/mL. For background staining, cells were incubated with a control ascites fluid as previously reported.[41] After 30-minute incubation on ice, cells were washed twice to remove unbound excess antibody and stained at 20×10^6 cells/mL with a dilution of 1:20 of FITC-labeled goat F(ab')$_2$ anti-mouse IgG (Cappel Laboratories, Cochranville, NC) for 45 minutes on ice. Subsequently, cells were washed three times to remove unbound antibody and immediately analyzed for IF using a modified FACS 440. An argon laser (400 mW, 488 nm) was used for excitation of FITC. Fluorescence emission for FITC was detected by selectively collecting 530 ± 15 nm. Low-angle forward-lightscatter and green fluorescence data were stored in the list mode for reanalysis by a Consort 40 PDP/11 computer system (Becton Dickinson, FACS Division) as described [55] During analysis of the list mode data files, lymphoid cells were discriminated from monocytes and dead cells by their characteristic low-angle forward-lightscatter properties. Sterile cell sorting was performed to isolate viable and CD19$^+$/CD10$^+$ select lymphoid cells. Only cells exhibiting fluorescence intensity clearly above background were sorted into the positive fraction at 1,500 cells/s. Sorted cells were 95% to 100% TdT$^+$, <0.5% T-cocktail$^+$, <0.5% sIg$^+$, and >99.5% CD19$^+$/CD10$^+$. These virtually pure populations of leukemic B cell precursors were cultured in the presence of various concentrations of HMW-BCGFs and LMW-BCGFs for 7 days under the conditions of a recently described B cell precursor colony assay system,[41] as detailed hereinafter.

B Cell precursor colony assay system Freshly obtained, density gradient-separated, adherent cell-depleted, and highly blast-enriched (>90%) leukemic B cell precursors from all 28 patients were assayed for in vitro colony formation using a B cell precursor colony assay system.[41] In 13 of these 28 cases, we also used FACS-sorted, virtually pure populations of CD19$^+$/CD10$^+$ marrow blasts. Only a brief description of the assay system will be given here, inasmuch as the detailed methodology has been published in earlier reports.[41,56] Leukemic B cell precursor blasts were suspended in α-MEM (minimal essential medium) supplemented with 0.9% methylcellulose, 30% calf bovine serum, various concentrations of either partially purified HMW-BCGFs, LMW-BCGFs, both GFs, or 10% (vol/vol) PHA-LCM (positive controls), or no added exogeneous BCGFs (negative controls), 1% MEM vitamin solution, 0.5% MEM amino acids solution, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 50 μmol/L 2-mercaptoethanol, 4 μg/mL D-L-serine, and 4 μg/mL asparagine. Replicate 1-mL samples containing 10^5 blasts were cultured in 35-mm Petri dishes for 7 days at 37°C in a humidified 5% CO$_2$ atmosphere. On day 7, colonies containing >20 cells were counted on a grid using an inverted-phase microscope with high optical resolution. Subsequently, colonies were harvested for further morphological, immunologic, and cytogenetic analysis of colony cells. The morphology of cells was studied in Wright's Giemsa–stained cytospin preparations. The surface marker profiles were analyzed on acetone-fixed cytospin slides by indirect IF as previously described[38] using BA-3 (anti-CD10/CALLA), B43 (anti-CD19), Leu14 (anti-CD22), and BA-1 (anti-CD24) mAb. FITC-conjugated goat F(ab')$_2$ anti-mouse IgG was used as secondary antibody. Cell sIg was tested by direct IF with FITC-conjugated goat anti-human total Ig (Kallestad). Cells were evaluated for cytoplasmic μ chains using FITC-conjugated F(ab')$_2$ goat anti-human IgM heavy chain-specific anti-μ Nuclear terminal deoxynucleotidyl transferase (TdT) activity of colony blasts was determined by indirect IF on methanol-fixed cytospin preparations using immunoadsorbent-purified rabbit anti-TdT serum and FITC-conjugated goat F(ab')$_2$ anti-rabbit IgG as described.[42,56] The percentage of cells expressing each marker was determined using a Zeiss fluorescent microscope equipped with Ploem epi-illumination. Quantitative data were obtained from examination of 100 to 200 cells Banded chromosome analyses were performed on colony blasts using a modification of our standard procedure.[46] In brief, 1×10^6 blasts from pooled colonies were cultured in flat-bottom tissue culture plates for 17 hours at 37°C in a humidified 5% CO_2 atmosphere in the presence of 10^{-7} mmol/L amethopterin. Subsequently, thymidine (10^{-5} mmol/L) and adenosine (10 μg/mL) were added, and cells were incubated for 4 hours. Cells were then subjected to colcemid, transferred to siliconized centrifuge tubes, centrifuged, and resuspended in a prewarmed, hypotonic KCl solution (1.5 g KCl, 0.1 g EDTA, 2.4 g HEPES in 500 mL H_2O, pH 7.4, 37°C). After 55-minute incubation at 37°C, cells were fixed using a methanol/acetic acid solution (3:1), and slides were prepared by conventional techniques. G-banding was performed with Wright's stain. Twenty to 50 metaphases were completely analyzed, and 5 to 10 cells were photographed and karyotyped.

Statistical Analysis. Statistical analysis was performed in the log scale by using two-sample, two-sided Student's *t* tests. As in previous studies[50,55] groups have been compared in the log scale by analysis of variance

RESULTS

Clinical and diagnostic features of B cell precursor ALL patients. Twenty-eight patients with B cell precursor ALL were studied. Patient data are summarized in Table 1. The patients ranged in age from 1 to 51 years (median 7 years). Most patients (86%) were children or adolescents aged <16 years. There were 11 males and 17 females. Seven patients were in relapse, and 21 were newly diagnosed. The WBC at the time of diagnosis varied from 900 to 246,000/μL (median 50,000/μL). Fourteen patients (50%) had a WBC of >50,000/μL. Twenty-three patients (82%) were morphologically classified as L1-ALL; the subtype of the remaining 5 was L2. Bone marrow aspirate samples evaluated in the present study contained 77% to 100% (median 95%) blasts.

Immunologic surface marker profiles of fresh marrow blasts. The immunologic profiles of freshly obtained leukemic marrow blasts are shown in Table 1. None of the patients expressed the T lineage-associated surface determinants CD2, CD5, or CD7. In contrast, 25 of the 28 patients (89%) expressed the B lineage-associated surface marker CD9, 27 (96%) expressed CD10/CALLA, 17 of 17 patients (100%) tested expressed the B lineage-restricted surface marker CD19, and all 28 patients (100%) expressed the B lineage-associated surface determinant CD24 but lacked sIg These immunologic marker profiles are consistent with the B cell precursor stage of human B lineage lymphoid differentiation.

Cytogenetic features of fresh bone marrow blasts Karyotype analyses were performed in 21 patients (Table 2). Four patients had a normal diploid karyotype (UPN 1, 17, 18, 26), 7 patients had a pseudodiploid karyotype (UPN 3, 5, 8, 21, 24, 25, 27), 6 patients had a near-diploid karyotype (45 or 47 chromosomes) with SCA (UPN 6, 7, 10, 11, 22, 28), 1 patient had a hypodiploid karyotype

Table 1. Clinical and Diagnostic Data on B Cell Precursor ALL Patients

						Immunological Marker Profiles							
UPN	Sex/Age	Diagnosis (FAB)	New/Relapse	WBC at Diagnosis	BM (% Blasts)	CD2 35 1	CD5 T101	CD7 3A1	CD9 BA-2	CD10 BA-3	CD19 B43	CD24 BA-1	sIg
1	M/10	L2	New	59,000	88	7	8	ND	76	87	95	59	6
2	M/18	L1	New	9,100	91	4	4	4	92	95	ND	96	4
3	M/9	L1	New	75,400	77	5	5	5	93	90	90	92	5
4	M/4	L1	New	43,400	94	3	3	4	40	75	95	81	2
5	F/4	L1	New	28,000	98	2	2	2	43	91	94	97	3
6	F/8	L1	New	1,700	98	2	2	2	33	90	ND	85	2
7	F/3	L1	New	107,400	89	1	1	0	74	93	ND	87	1
8	F/16	L1	New	76,400	96	7	2	2	34	33	ND	82	2
9	F/2	L1	New	4,900	100	2	2	2	95	94	43	94	2
10	M/6	L1	New	6,100	99	ND	3	9	42	79	91	96	1
11	F/3	L2	New	72,800	98	6	6	6	91	92	87	82	7
12	F/2	L1	New	45,200	99	10	10	10	87	80	77	75	6
13	M/7	L2	Relapse	77,000	95	2	2	ND	40	88	94	64	6
14	M/10	L1	Relapse	6,900	89	2	1	ND	82	37	97	83	0
15	F/7	L2	Relapse	73,000	81	2	1	2	5	64	ND	86	2
16	F/6	L1	New	50,700	99	4	7	6	81	80	60	84	5
17	F/14	L1	New	3,500	92	2	4	ND	86	17	ND	89	6
18	M/51	L1	New	5,000	95	2	5	2	92	96	91	97	2
19	F/12	L1	New	150,000	94	3	3	4	12	91	95	89	1
20	M/8	L1	New	16,700	95	3	2	3	89	93	80	86	1
21	M/41	L1	Relapse	150,000	86	6	5	5	86	86	97	90	6
22	F/6	L1	New	63,200	97	8	7	8	31	86	ND	90	5
23	F/6	L1	Relapse	3,400	98	4	4	3	79	95	ND	86	3
24	F/6	L2	Relapse	116,800	89	2	2	6	91	93	ND	97	2
25	F/2	L1	New	27,500	78	5	4	4	72	95	85	96	2
26	M/5	L1	Relapse	50,000	90	2	2	ND	4	93	ND	55	1
27	F/1	L1	New	89,900	91	3	3	3	75	33	76	91	7
28	F/14	L1	New	11,900	97	11	9	10	65	81	ND	82	6

The immunologic marker profiles of BM blasts were determined by indirect and direct IF and flow cytometry as described in the Materials and Methods section Results are expressed as the percentage of BM cells reactive with each marker antibody Abbreviations: UPN, unique patient number; ND, not determined

Table 2. Cytogenetic Features of B Cell Precursor ALL Patients

UPN	Karyotype of Freshly Obtained Bone Marrow Cells
1	46,XY
2	54,XY/55,XY/57,XY
3	46,XX/46,XX,t(12;18)(p12;q12)
4	46,XY/54,XY,+Y,+4,+6,+10,+14,+18,+21,t(9,22)(q34;q11),+der(22),t(9;22)(q34,q11)
5	46,XX,t(12;18)(p12;q12)
6	46,XX(45,XX,−7,−9,−9,−18,−21,+der(21),t(?7;21)(?q22,q22),+3mar
7	46,XX/47,XX,t(2,16)(p11,p11),+mar
8	46,XX/46,XX,−16,+mar
10	46,XY/47,XY,+mar
11	45,XX,t(7,12)(p22;q13)
13	46,XY/43,XY,−2,−9,−13,−14,−17,+der(2),t(2;9)(q33;q11),+der(17),t(17,?)(p13;?),+der(18),t(18;?)(q21?;?)/43,XY,−1,−2,−3,−9,−13,−14,−17,−18,del(1)(p22p36),t(4;20)(p16?;q11),+der(1),t(1,?)(p36,?),+der(2),t(2;9)(q33,q11),+der(17),t(17,?)(p13;?),+der(18),t(18;?)(q21?;?),+mar
14	46,XY/56,XY,+X,+4,+5,+11,+14,+15,+18,+19,+21,+22
17	46,XX
18	46,XY
21	46,XY,−15,+mar,t(9;22)(q34;q11)
22	46,XX/45,XX,−7,2p−
24	46,XX,t(6;12)(p21,p13),del(7)(p?13p?22)
25	46,XX,del(12)(p11),t(2;7)(?q11;?q11)
26	46,XY
27	46,XX/46,XX,t(5,15)(p15,q11)/47,XX,+22,t(5;15)(p15,q11)
28	45,XX,−17,−22,del(6)(q13q21),del(13)(q22),t(3;12)(q21;p13),+der(22);t(17,22)(q11,p12)

and multiple SCA (UPN 13), and 3 patients had a hyperdiploid karyotype (UPN 2, 4, 14). Fifteen patients had SCA (UPN 3, 4 through 8, 10, 11, 13, 21, 22, 24, 25, 27, 28). Among these 15, 5 had SCA involving 12p11-13 (UPN 3, 5, 24, 25, 28) and 2 had a Philadelphia chromosome (UPN 4, 21).

Expression of BCGF receptors on fresh leukemic marrow lymphoblasts in B-cell precursor ALL. To study the constitutive expression of BCGF receptors at the B cell precursor stage, we first investigated the binding of ^{125}I-HMW-BCGF (0.8×10^{18} cpm/mol) to freshly obtained marrow blasts from 15 B cell precursor ALL patients (Table 3). A marked interpatient variation in the amount of specific binding of radioiodinated HMW-BCGF to leukemic marrow B cell precursors was observed. Among the 15 cases studied, the estimated number of ^{125}I-HMW-BCGF molecules bound per blast ranged from "undetectable" to 24.3×10^3 (median 3.5×10^3). Because of the limited amounts of highly purified BCGF, cold competition experiments were performed using only a tenfold excess of unlabeled ligands. Therefore, the values for percentage of inhibitable binding were relatively low in some cases. The proliferative response of fresh marrow blasts to exogenous HMW-BCGF was determined in standard ^{3}H-TdR incorporation assays to assess the function of the detected HMW-BCGF receptors. In 5 of 15 cases (33%), incubation with HMW-BCGF (5 ng/mL) resulted in a 1.3 (UPN 25) to 4.4-fold (UPN 9) increase of ^{3}H-TdR incorporation by leukemic B cell precursor blasts. Ten of 15 cases failed to respond, despite the fact that in four of these patients (UPN 1, 3, 11, 13), marrow blasts expressed a significant number of HMW-BCGF receptors binding an average $>3.5 \times 10^3$ ^{125}I-HMW-BCGF molecules/cell. In side-by-side experiments, we studied the specific binding of ^{125}I-LMW-BCGF (0.8×10^{17} cpm/mol) to fresh leukemic B cell precursor marrow blasts. The estimated number of radioiodinated LMW-BCGF molecules bound per cell ranged from 11.5×10^3 (UPN 1) to 457.8×10^3 (UPN 13) (median 37.4×10^3; UPN 10), exceeding in seven of eight cases the number of bound radioiodinated HMW-BCGF molecules per cell under identical conditions by 2.2 (UPN 1) to 100.0-fold (UPN 20).

We studied the biologic effects of LMW-BCGF on fresh leukemic B cell precursor blasts in 16 cases. In 12 cases (75%), leukemic B cell precursors responded to LMW-BCGF (2 ng/mL) with increased ^{3}H-TdR incorporation, and the stimulation indices varied from 1.5 (UPN 15) to 33.9 (UPN 10). In 6 (UPN 9, 10, 11, 15, 21, 25) of 14 cases compared, the bulk of leukemic marrow B cell precursors responded to both HMW-BCGF and LMW-BCGF. In four cases, (UPN 1, 3, 8, 15), no proliferative response to either form of BCGF was noted, and in the remaining four cases (UPN 13, 14, 19, 20), marrow blasts responded only to LMW-BCGF but not to HMW-BCGF. In parallel experiments, fresh marrow blasts were cultured in the presence of 5 ng/mL HMW-BCGF or 2 ng/mL LMW-BCGF for 72 hours; the immunophenotype of viable cells was then determined by IF on cytospin slides as described.[41] In both HMW-BCGF and LMW-BCGF cultures, the viable cells were surface B43$^+$, cytoplasmic CD22$^+$, but lacked sIg, confirming that the increase of ^{3}H-TdR incorporation was not due to increased DNA synthesis by contaminating sIg$^+$ normal B lymphocytes (data not shown).

Expression of BCGF receptors on leukemic B lineage lymphoid marrow progenitor cells. Because of the inherent limitations of ^{3}H TdR incorporation assays,[57] measurements of the amounts of ^{3}H-TdR may not be accurate as quantitative indicators of leukemic B cell proliferation in the presence of BCGF. Therefore, it was important to evaluate the stimulatory effects of HMW-BCGF and LMW-BCGF in a clonogenic assay system. B cell precursor colony assays were performed using highly blast-enriched, fresh bone marrow samples from 28 patients with untreated, newly diagnosed or relapsed B cell precursor ALL to assess and compare the biologic effects of purified natural BCGFs on leukemic B lineage lymphoid progenitor cells. Table 4 illustrates the proliferative responses of fresh leukemic lymphoid progenitor cells from B cell precursor ALL patients to HMW-BCGF and LMW-BCGF. Controls included cultures with no exogenous BCGF (negative background controls) and cultures with 10% (vol/vol) PHA-LCM containing several growth factors including both HMW-BCGF and LMW-BCGF (positive controls). Of 28 cases studied, 12 did not respond to HMW-BCGF, whereas in the remaining 16 cases (57%), HMW-BCGF resulted in a marked stimulation of leukemic progenitor cells (Table 4). In 10 cases (UPN 4, 5, 7, 11, 14, 16, 21, 24, 25, 28), we observed a dose-depen-

Table 3. Expression and Functional Integrity of BCGF Receptors in B Cell Precursor ALL

	Specific ^{125}I-HMW-BCGF Binding			Specific ^{125}I-LMW-BCGF Binding			Stimulation Index	
UPN	Inhibitable Binding (%)	cpm	Molecules/Cell × 10^3	Inhibitable Binding (%)	cpm	Molecules/Cell × 10^3	HMW-BCGF	LMW-BCGF
1	21	700	5.3	5	153	11.5	1 0	0.6
3	18	898	6.7	ND	ND	ND	1.0	1.0
8	40	169	1 3	ND	ND	ND	1.0	0.8
9	14	480	3 6	ND	ND	ND	4.4	2 0
10	5	160	1 2	27	498	37.4	1 0	33.9
11	44	3,240	24.3	18	298	22 4	1.5	1.6
13	24	990	7 4	84	6,104	457.8	1.0	9 1
14	19	690	5.2	45	880	66.0	1.0	4.6
15	0	0	0 0	ND	ND	ND	1 0	1.5
16	5	130	1.0	ND	ND	ND	1 3	1.0
17	28	1,140	8.6	ND	ND	ND	3.1	ND
18	ND	ND	ND	39	896	67.2	ND	14 5
19	1	30	0.2	ND	ND	ND	1.0	5.2
20	13	460	3.5	87	4,670	350.3	1.0	13.8
21	21	760	5.7	10	208	15 6	2.7	13.8
25	11	360	2.7	5	154	11 6	1.3	2.1

BCGF receptor binding and receptor function studies were performed as described in the Materials and Methods section. Binding data are expressed as percentage of inhibitable binding, total cell associated cpm inhibitable by excess cold BCGF, and the estimated number of radioiodinated BCGF molecules bound per cell. Stimulation indices were determined in ^{3}H-TdR incorporation assays as described in the Materials and Methods section.

Table 4. Proliferative Responses of Fresh Leukemic Lymphoid Progenitor Cells From B Cell Precursor ALL Patients to Purified Natural Human BCGF

		Mean No. of B Lineage Blast Colonies per 10^5 Unsorted Fresh Marrow Blasts						
		HMW-BCGF (ng/mL)			LMW-BCGF (ng/mL)			
UPN	No BCGF	1.0	2.0	5 0	1 0	2 0	5 0	PHA-LCM 10% (v/v)
1	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0
4	0	50	203	510	250	814	507	1,153
5	0	74	690	1,504	389	580	504	2,042
6	0	0	0	0	44	280	35	266
7	0	97	295	375	285	334	351	1,154
8	0	0	0	0	0	0	0	0
9	0	0	0	150	1	80	53	0
10	0	0	0	0	700	1,250	100	0
11	0	0	55	230	30	176	0	133
12	0	0	0	198	35	250	0	0
13	15	10	25	97	82	390	20	140
14	75	250	1,554	1,987	195	500	544	350
15	0	5	174	12	0	24	27	0
16	0	3	125	202	0	14	3	236
17	0	0	0	0	0	0	0	0
18	50	0	58	246	104	502	496	146
19	30	17	10	5	296	378	350	311
20	105	98	100	54	250	1,037	1,155	446
21	25	50	195	294	185	1,047	1,233	181
22	0	0	0	0	50	230	170	400
23	0	0	0	0	125	190	204	251
24	0	185	464	1,280	280	940	800	645
25	0	0	187	241	132	365	0	515
26	0	0	0	0	0	0	0	0
27	0	0	0	125	0	280	274	210
28	0	280	715	1,400	568	2,354	367	1,235

The proliferative responses of leukemic B lineage lymphoid progenitor cells to BCGF were determined in colony assays as described in the Materials and Methods section Results are expressed as the mean number of B lineage blast colonies per 10^5 cells plated PHA-LCM 10% (vol/vol) was used as a positive control.

dent increase in the number of B lineage blast colonies in response to HMW-BCGF and in 8 of these 10 cases (all except UPN 11 and 25), colony numbers were clearly above background even at 1 ng/mL factor. By comparison, in five cases (UPN 9, 12, 13, 18, 27), we obtained stimulation of blast colony formation only at 5 ng/mL HMW-BCGF. In all but one patient (UPN 15), 5 ng/mL HMW-BCGF yielded the maximum mean number of colonies. This number, however, showed a marked interpatient variation, ranging from 97 colonies (UPN 13) to 1,987 colonies (UPN 14) per 10^5 blasts plated (mean 551 colonies/10^5 blasts). We also observed a pronounced interpatient variability in proliferative response of leukemic progenitor cells to LMW-BCGF. Six cases (UPN 1 through 3, 8, 17, 26) failed to respond, and two (UPN 15, 16) had very low colony numbers. In the remaining 20 cases, B lineage blast colony numbers in LMW-BCGF–stimulated cultures were significantly higher than the background. In 15 (UPN 4 through 6, 9, 10 through 13, 18, 19, 22, 24, 25, 27, 28) of these 20 cases, maximum stimulation was achieved with 2 ng/mL LMW-BCGF whereas in 5 cases (UPN 7, 14, 20, 21, 23), maximum colony numbers were observed in cultures stimulated with 5 ng/mL LMW-BCGF (Table 4). The maximum colony numbers varied from 14 (UPN 16) to 2,354 (UPN 28) per 10^5 blasts plated. Among the 28 cases evaluated in colony assays, 16 (57%) responded to both HMW-BCGF and LMW-BCGF, 6 (21%) responded only to LMW-BCGF and the remaining cases showed no proliferative response to either GF. We did not observe any instance in which leukemic progenitor cells responded to HMW-BCGF but failed to respond to LMW-BCGF. Of the 16 cases that responded to both forms of BCGF, 7 yielded more colonies with HMW-BCGF (UPN 5, ,9, 11, 14 through 16, 24), 8 yielded more colonies with LMW-BCGF (UPN 4, 12, 13, 18, 21, 25, 27, 28), and in one case both forms were equally effective (UPN 7). Notably, in HMW-BCGF– or LMW-BCGF–stimulated cultures, lymphoid progenitor cell colonies displayed a marked variability in size, with cell numbers ranging from 50 to >500 cells/colony (data not shown). To reach a colony size of 500 cells within 7 days of culture, leukemic B cell precursor blasts had to undergo more than eight cell cycles, with an average doubling time of 19 hours [$2^n = 500$ cells, where n = number of cell cycles; $n = 8.97$; 7 (days of culture) × 24 (h/day)/n (number of cell cycles per 7 days culture period) = doubling time = 18.73 hours]. This rapid expansion of leukemic B cell precursor ALL blasts in the presence of BCGFs is direct and unambiguous evidence that both HMW-BCGF and LMW-BCGF stimulate proliferation of leukemic B cell precursors. Consistent with our pervious reports,[38,54] PHA-LCM supported B lineage blast colony formation in 18 of 28 cases (64%) studied (Table 4).

Comparison of BCGF responses of fresh leukemic marrow B cell precursors v *their progenitor blasts.* By comparing the data on 14 evaluable patients from Tables 3 and 4, we identified four groups based on HMW-BCGF response of leukemic B cell precursors as determined by ^{3}H-TdR incorporation assays, and the response of their progenitor blasts as determined by colony assays. In the first group of seven cases (UPN 1, 3, 8, 10, 16, 19, 20), neither the bulk population of fresh marrow blasts nor their colony-forming progenitor cells responded to HMW-BCGF. In the second group of two cases (UPN 11, 21), both fresh marrow blasts as well as their progenitors showed a proliferative response to HMW-BCGF. In the third group of two cases (UPN 9, 17), fresh marrow blasts but not their progenitors responded to HMW-BCGF, whereas in the fourth group of three cases (UPN 13 through 15), only the progenitor cells showed a detectable BCGF response. In contrast to HMW-BCGF response, the proliferative response of the bulk of leukemic marrow B cell precursors to LMW-BCGF correlated with the LMW-BCGF response of their progenitor cells. In 4 (UPN 1, 3, 8, 16) of 15 cases analyzed, neither the bulk of leukemic marrow B cell precursors nor their progenitor blasts showed a significant response to LMW-BCGF. In comparison, in the remaining 11 cases, both the bulk of marrow blasts and their progenitor cells showed a marked response. The lowest proliferative response among these 11 cases was observed in UPN 15 with a stimulation index of 1.5 for ^{3}H-TdR incorporation and a mean of only 27 B lineage blast colonies/10^5 cells cultured in LMW-BCGF–stimulated samples. The highest poliferative response was observed in UPN 10, with a stimulation index of 33.9 for ^{3}H-TdR incorporation and a mean of 1,250 blast colonies/10^5 cells cultured in LMW-BCGF–stimulated samples.

Morphological, Immunologic and cytogenetic features of colony blasts in BCGF-stimulated cultures In all 22 cases that responded to either HMW-BCGF, LMW-BCGF, or both, cells pooled from unselected colonies growing in HMW-BCGF– or LMW-BCGF–stimulated cultures were analyzed in detail to confirm their leukemic origin and B lineage affilliation. Wright's-Giemsa–stained colony cells had a lymphoblast morphology that differed from the appearance of the bulk of preculture marrow blasts in that the former had a bigger size, more prominent vacuolation, more prominent nucleoli, and a more irregular nuclear membrane. A broad panel of mAbs were used to study the immunologic marker profiles of colony blasts. As shown in Table 5, pooled colony blasts were nuclear TdT$^+$ (22 of 22), surface CD10/CALLA$^+$ (17 of 22), CD19$^+$ (22 of 22), cytoplasmic CD22$^+$ (21 of 22), surface CD24$^+$ (22 of 22), but lacked sIg (22 of 22) or cytoplasmic μ heavy chains (21 of 22). In addition, cells did not react with 3A1 mAb directed against the T lineage marker CD7 (data not shown). These immunophenotypic features are consistent with B cell precursor ALL. Colony blasts from only one case (UPN 20, pre-B ALL) were cytoplasmic μ^+, consistent with pre-B ALL (uncultured bone marrow blasts from this patient were also cytoplasmic μ^+). Karyotypic analyses of colony blasts were performed in five cases with documented cytogenetic abnormalities before culture (UPN 4, 13, 14, 21, 24) and, in one case, with a normal diploid karyotype (UPN 18). As detailed in Table 6, colony blasts from BCGF-stimulated cultures in UPN 4, 13, 14, and 24 had clonal cytogenetic abnormalities identical (UPN 4, 13) or closely related (UPN 14, 24) to the karyotype of uncultured marrow blasts. In UPN 18, initial marrow blasts as well as colony blasts in BCGF-stimulated cultures had a normal diploid karyotype. In contrast, in UPN 21, the cytogenetic features of colony blasts (ie, hyperdiploid

Table 5. Immunologic Marker Profiles of Colony Blasts in BCGF-Stimulated Cultures

UPN	Nuclear TdT	Surface CALLA/CD10 BA-3	Surface CD19 B43	Cytoplasmic CD22 Leu 14	Surface CD24 BA-1	Cytoplasmic μ	Surface Ig
4	100	50	50	34	75	0	0
5	95	50	80	75	80	0	0
6	100	58	62	54	50	0	0
7	100	58	75	64	90	0	0
9	91	65	94	50	75	0	0
10	100	72	85	37	92	0	0
11	89	78	75	69	90	2	0
12	100	14	69	12	52	0	0
13	100	85	95	75	68	0	0
14	90	5	93	78	44	0	0
15	100	85	91	63	77	3	0
16	92	50	84	35	47	0	0
18	85	72	72	55	81	4	0
19	100	5	78	65	77	0	0
20	98	75	80	35	45	32	2
21	97	1	65	60	78	0	0
22	88	57	92	58	61	4	0
23	100	52	62	59	75	0	0
24	85	100	99	75	100	0	0
25	94	27	74	60	85	1	0
27	100	0	86	75	54	0	0
28	93	75	69	39	75	3	1

The immunophenotypes of B lineage colony blasts were determined by IF staining techniques and IF microscopy as described in the Materials and Methods section. Results are expressed as the percentage of cells reactive for each marker. Quantitative data were obtained from examination of 100 to 200 blasts.

with SCA) were different from those of uncultured marrow blasts (ie, pseudodiploid) Notably, colony blasts were CALLA negative (Table 5), although 86% of uncultured marrow blasts were CALLA positive (Table 1). These findings suggest that the known CALLA-positive, pseudodiploid clone in UPN 21 did not respond to HMW-BCGF or LMW-BCGF, whereas the clonogenicity of a previously undetected CALLA-negative, hyperdiploid clone was increased by BCGF stimulation, allowing its detection by karyotypic analysis. This case will be the subject of future studies in our laboratory to confirm its biclonality. Despite the observed differences between preculture marrow blasts and BCGF-stimulated cultured blasts, colony blasts in all analyzed cases with documented chromosomal abnormalities had cytogenetic abnormalities that confirmed their leukemic origin. In summary, colony blasts from BCGF-stimulated cultures had morphological, immunologic, and cytogenetic features consistent with B cell precursor ALL.

Table 6. Cytogenetic Features of Colony Blasts From BCGF-Stimulated Cultures

UPN	Karyotype of Abnormal Clone(s)	
	Fresh Bone Marrow	BCGF-Stimulated Cultures
4	54,XY,+Y,+4,+6,+10,+14,+18,+21,t(9;22)(q34;q11),+der(22),t(9;22)(q34;q11)	54,XY,+Y,+4,+6,+10,+14,+18,+21,t(9;22)(q34;q11),+der(22),t(9;22)(q34;q11)
13	43,XY,−2,−9,−13,−14,−17,+der(2),t(2;9)(q33;q11),+der(17),t(17;?)(p13;?),+der(18),t(18;?)(q21?;?)/43,XY,−1,−2,−3,−9,−13,−14,−17,−18,del(1)(p22p36),t(4;20)(p16?;q11);+der(1),t(1;?)(p36;?),+der(2),t(2;9)(q33;q11),+der(17),t(17;?)(p13,?),+der(18),t(18;?)(q21?;?),+mar	43,XY,−Y,−1,−2,−9,−14,−18,del(1)(p22p36),+der(1),t(1;?)(p36,?),+der(2),t(2;9)(q33;q11),der(11),t(11;?)(p14;?),+der(18),t(18;?)(q2?1;?)
14	56,XY,+X,+4,+5,+11,+14,+15,+18,+19,+21,+22	56,XY,+X,+4,+6,+8,+15,+18,+18,+21,+21,+22,dup(1)(q2?1→q4?4)
18	No abnormal clone, 46,XY	No abnormal clone; 46,XY
21	46,XY,−15,t(9;22)(q34;q11),+mar	52,XY,+X,+19,+20,t(2;14)(p11;q32),t(9;22)(q34;q11),+der(22)t(9;22)(q34;q11)+2 mar
24	46,XX,t(6;12)(p21;p13),del(7)(p?,13p?22)	46,XX,t(6;12)(p21;p13),del(7)(p13p22)/46,XX,t(6;12)(p21;p13)

Banded chromosome analyses were performed as described in the Materials and Methods section. In each case, 20 to 50 metaphases were analyzed, and 5 to 10 cells were karyotyped. An abnormal clone was defined as >2 metaphase cells with identical SCA, or identical extra chromosomes, or >3 metaphases with identical missing chromosomes

Expression of BCGF receptors on CD19$^+$/CALLA$^+$ FACS-sorted fresh leukemic B lineage lymphoid progenitor cells. Our next concern was whether the observed modulation of proliferative activity of leukemic B lineage lymphoid progenitor cells was directly mediated by BCGF or indirectly mediated by soluble GF released from "accessory" cells. To address this question, the proliferative responses of leukemic lymphoid progenitor cells to HMW-BCGF and LMW-BCGF were also evaluated using virtually pure populations of fresh CD19$^+$/CALLA$^+$, FACS-sorted leukemic blasts from 13 B cell precursor ALL patients (Table 7). In accordance with the first series of experiments summarized in Table 4, the observed effects of BCGF were dose dependent, and optimal stimulation of FACS-sorted leukemic B lineage lymphoid progenitor cells was achieved at 5 ng/mL HMW-BCGF or 2 ng/mL LMW-BCGF. In successfully cultured cases, the mean maximum number of B lineage blast colonies per 10^5 FACS-sorted blasts (Table 7) was usually higher than the mean number of blast colonies per 10^5 unsorted blasts (Table 4), probably due to either further enrichment by FACS, removal of some accessory cells that may have an inhibitory effect on leukemic B lineage lymphoid progenitor cells, or both. As with unsorted blasts (Table 4), we observed a marked interpatient variability in proliferative response of FACS-sorted leukemic B cell precursors to BCGF (Table 7). In nine cases (UPN 4, ,5, 11, 13, 14, 18, 21, 24, 25), blast progenitors responded to both HMW-BCGF and LMW-BCGF, whereas in two patients (UPN 1, 3), neither HMW-BCGF nor LMW-BCGF promoted blast colony formation. In the remaining two cases (UPN 20, 22), blast colonies were observed only in LMW-BCGF–stimulated samples. These findings establish that both HMW-BCGF and LMW-BCGF exert a direct stimulatory effect on leukemic B lineage lymphoid progenitor cells, which represent the earliest B lineage lymphoid cells detectable by currently available techniques.

Synergy between HMW-BCGF and LMW-BCGF at the level of leukemic B lineage lymphoid progenitor cells. We next studied the combined effects of HMW-BCGF and LMW-BCGF on leukemic B lineage lymphoid progenitor cells using CD19$^+$/CALLA$^+$, FACS-sorted fresh marrow blasts from eight B cell precursor ALL patients (Table 8). Synergistic effects were observed in four (UPN 4, 13, 14, 21) of five cases that responded to both HMW-BCGF and LMW-BCGF (ie, the number of blast colonies in cultures stimulated with HMW-BCGF in combination with LMW-BCGF was greater than the sum of the numbers of blast colonies obtained with HMW-BCGF or LMW-BCGF alone, and this difference was statistically significant with $P < .05$, as determined by standard two-sample, two-sided t tests in the log scale), whereas in the remaining case (UPN 24), the combined effects were less than additive at any BCGF concentration tested. In UPN 13 and 21, a combination of 1 or 2 ng/mL HMW-BCGF with 1 or 2 ng/mL LMW-BCGF yielded a synergistic increase of leukemic B cell precursor colony numbers. In comparison, in UPN 4 and 14, synergistic effects between HMW-BCGF and LMW-BCGF were observed only when they were mixed at 1 ng/mL of each. The increase in colony numbers was less than additive in UPN 4 and 14 when 2 ng/mL HMW-BCGF was combined with 2 ng/mL LMW-BCGF. In UPN 18 and 22, responding only to LMW-BCGF, the combined effects of HMW-BCGF and LMW-BCGF were less than additive regardless of the BCGF concentrations used. Lymphoid progenitor cells obtained from UPN 1 did not respond to HMW-BCGF, LMW-BCGF, or a combination of both.

Associations between BCGF response of leukemic lymphoid progenitor cells and other laboratory or clinical parameters. Table 9 details the correlations between various diagnostic parameters of B cell precursor ALL patients and the BCGF response of their freshly obtained leukemic lymphoid progenitor cells. The response of leukemic progenitor cells to HMW-BCGF or LMW-BCGF did not correlate to sex, age, disease status (newly diagnosed *v* relapsed), FAB morphology, or WBC at diagnosis (Table 9). Furthermore, no statistically significant associations emerged when we compared the BCGF response of leukemic progenitor cells from B cell precursor ALL patients with respect to expres-

Table 7. Proliferative Responses of B43$^+$/CALLA$^+$ FACS-Sorted Fresh Leukemic Lymphoid Progenitor Cells From B Cell Precursor ALL Patients to Purified Natural Human BCGF

		Mean No of B Lineage Blast Colonies per 10^5 CD19$^+$/CD10$^+$ FACS-Sorted Fresh Marrow Blasts							
		HMW-BCGF (ng/mL)				LMW-BCGF (ng/mL)			
UPN	No BCGF	0.2	1.0	2.0	5.0	0.2	1.0	2.0	5.0
1	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0
4	0	0	115	465	724	0	375	984	950
5	0	0	272	1,375	2,104	124	280	730	682
11	0	0	0	144	415	0	43	246	0
13	25	28	26	123	184	0	138	480	0
14	111	113	182	2,233	2,589	142	366	1,114	ND
18	74	0	54	71	384	48	119	449	0
20	92	84	90	94	89	212	307	1,228	ND
21	19	21	74	244	320	136	293	1,388	ND
22	0	0	0	0	0	14	72	194	198
24	0	0	150	502	1,400	0	240	1,015	ND
25	0	0	0	225	357	0	201	469	ND

Cell sorting and colony assays were performed as described in the Materials and Methods section. Results are expressed as the mean number of B lineage ALL blast colonies per 10^5 FACS-sorted, CD19$^+$/CD10$^+$ fresh marrow blasts. ND, not determined.

Table 8. Synergy Between HMW-BCGF and LMW-BCGF at the Level of Leukemic B Lineage Lymphoid Progenitor Cells

UPN		HMW-BCGF (ng/mL)	LMW-BCGF (ng/mL)	Mean No. of Blast Colonies per 10^5 Cells	UPN		HMW-BCGF (ng/mL)	LMW-BCGF (ng/mL)	Mean No. of Blast Colonies per 10^5 Cells
1	Sample 1	0.0	0.0	0	18	Sample 1	0.0	0 0	[illegible]74
	Sample 2	1.0	0.0	0		Sample 2	1.0	0.0	[illegible]54
	Sample 3	0.0	1.0	0		Sample 3	0 0	1.0	[illegible]19
	Sample 4	1.0	1.0	0		Sample 4	1.0	1 0	[illegible]21
	Sample 5	2.0	0.0	0		Sample 5	2 0	0.0	[illegible]71
	Sample 6	0.0	2.0	0		Sample 6	0.0	2.0	[illegible]49
	Sample 7	2.0	2.0	0		Sample 7	2.0	2 0	[illegible]52
4	Sample 1	0.0	0.0	0	21	Sample 1	0.0	0 0	19
	Sample 2	1.0	0.0	115		Sample 2	1.0	0.0	74
	Sample 3	0.0	1.0	375		Sample 3	0 0	1.0	[illegible]93
	Sample 4	1.0	1.0	1,015		Sample 4	1 0	1.0	[illegible]88
	Sample 5	2.0	0.0	465		Sample 5	2.0	0.0	[illegible]44
	Sample 6	0.0	2.0	984		Sample 6	0.0	2.0	1,[illegible]88
	Sample 7	2 0	2 0	1,014		Sample 7	2 0	2.0	2,[illegible]02
13	Sample 1	0.0	0.0	25	22	Sample 1	0 0	0.0	0
	Sample 2	1.0	0.0	26		Sample 2	1 0	0.0	0
	Sample 3	0.0	1 0	138		Sample 3	0 0	1.0	72
	Sample 4	1.0	1.0	224		Sample 4	1.0	1 0	81
	Sample 5	2.0	0.0	123		Sample 5	2.0	0 0	0
	Sample 6	0 0	2 0	480		Sample 6	0 0	2.0	94
	Sample 7	2.0	2.0	744		Sample 7	2.0	2.0	98
14	Sample 1	0.0	0.0	111	24	Sample 1	0.0	0.0	0
	Sample 2	1.0	0 0	182		Sample 2	1 0	0.0	50
	Sample 3	0.0	1.0	366		Sample 3	0.0	1 0	[illegible]40
	Sample 4	1.0	1.0	2,750		Sample 4	1.0	1 0	[illegible]65
	Sample 5	2.0	0 0	2,233		Sample 5	2 0	0.0	[illegible]02
	Sample 6	0.0	2.0	1,114		Sample 6	0.0	2.0	1,[illegible]15
	Sample 7	2.0	2.0	2,803		Sample 7	2.0	2.0	1,300

FACS-sorted fresh marrow blasts from eight patients were used to study the combined effects of HMW-BCGF and LMW-BCGF. Each case was analyzed separately in an independent experiment. Results from eight experiments are shown as the mean number of blast colonies per 10^5 cells in replicate cultures in each experiment. The combined effects were described as synergistic when they were greater than additive at a given concentration.

Table 9. BCGF Response of Leukemic B Lineage Lymphoid Progenitor Cells

		B Cell Precursor Colony Formation			
		HMW-BCGF		LMW-BCGF	
Variables	No of Cases	Mean No. of Blast Colonies (±SE)	*P*	Mean No of Blast Colonies (±SE)	*P*
M	11	294 ± 176	NS	535 ± 155	NS
F	17	347 ± 124		383 ± 136	
Age ≥16 yr	4	135 ± 79	NS	434 ± 292	NS
Age <16 yr	24	358 ± 116		444 ± 111	
Relapse	7	547 ± 293	NS	477 ± 176	NS
New	21	252 ± 423		431 ± 124	
L2	5	356 ± 234	NS	307 ± 173	NS
L1	23	319 ± 114		472 ± 118	
WBC ≥50,000	14	200 ± 89	NS	287 ± 100	NS
WBC <50,000	14	453 ± 178		598 ± 171	
SCA	15	404 ± 139	NS	616 ± 161	0.04*
No SCA	6	372 ± 325		174 ± 110	
SCA involving 12p11–13	5	885 ± 316		848 ± 406	
Philadelphia chromosome positive	2	402 ± 108	0.02*	1,024 ± 210	NS
Other SCA	8	103 ± 49		370 ± 132	

Statistical analyses were performed as described in the Materials and Methods section

*Statistically significant.

sion of B lineage surface determinants, CD9, CD10/CALLA, CD19, or CD24 on their fresh marrow blasts (Tables 1 and 4, statistical data not shown). Of particular note is the fact that a pronounced association was seen between the BCGF response and the karyotypic findings (Table 9). Five patients had SCA involving the p11-13 region of the short arm of chromosome 12. In these patients, the proliferative response of leukemic progenitor cells to HMW-BCGF (885 colonies/10^5 cells) was significantly higher than in patients with a Philadelphia chromosome (402 colonies/10^5 cells), patients with other types of SCA (103 colonies/10^5 cells), or patients with no detectable SCA (372 colonies/10^5 cells, $P < .05$). In both Philadelphia chromosome-positive cases, the response to HMW-BCGF was greater than in cases without SCA. Because patients with SCA other than an aberration of 12p11-13 or a Philadelphia chromosome had significantly less leukemic B cell precursor colonies, however, than did patients without SCA, there was no statistically significant difference in HMW-BCGF response of leukemic progenitor cells from patients with SCA (404 colonies/10^5 cells) *v* patients without SCA (372 colonies/10^5 cells, $P > .1$). In comparison, the LMW-BCGF response of leukemic progenitor cells was significantly greater for patients with any form of SCA than for patients without SCA (616 colonies/10^5 cells *v* 174 colonies/10^5 cells, $P < .05$). Among patients with SCA, the mean number of leukemic B cell precursor colonies in LMW-BCGF–stimulated cultures, in contrast to HMW-BCGF–stimulated cultures, was not significantly greater in patients with an abnormality of 12p11-13 (848 colonies/10^5 cells) or with a Philadelphia chromosome (1,024 colonies/10^5 cells) than in patients with other types of SCA (370 colonies/10^5 cells, $P > .1$) The proliferative responses of leukemic progenitor cells from patients with an abnormality of 12p11-13 to HMW-BCGF and LMW-BCGF were almost identical (885 *v* 848 colonies/10^5 cells). In comparison, patients with SCA not involving 12p11-13 responded to LMW-BCGF with much higher colony numbers than to HMW-BCGF (501 colonies *v* 163 colonies/10^5 cells, $P < .05$).

DISCUSSION

Currently, very little is known about BCGF receptor expression or biologic effects of BCGF in early stages of B lineage differentiation. In this study, we analyzed expression of functional BCGF receptors at the level of the most immature human B lineage lymphoid cells detectable by currently available techniques. Our findings provide the first direct evidence of BCGF receptor expression at the B cell precursor stage of B lineage lymphoid differentiation.

The immunologically distinct types of human ALL[39,58] appear to reflect sequential stages of early lymphocyte ontogeny. Most ALL cases are represented by B cell precursor ALL patients who have immunologic surface marker profiles indicative of B lineage affiliation,[58-61] as well as rearrangements for Ig heavy and light chain genes.[62,63] Therefore, B cell precursor ALL is believed to originate from normal B cell progenitors allegedly arrested at discrete stages of differentiation within the B precursor pathway. Although numerous studies on B cell precursor ALL cells[60-64] have demonstrated that distinct stages of B lineage differentiation can be defined by expression of B cell-restricted and B cell-associated antigens, the biologic features of corresponding leukemic B lineage lymphoblasts have not been adequately analyzed. Critical information regarding surface GF receptors on B cell precursor ALL blasts and their putative normal counterparts has not been reported. The presumptive first step in GF action is the binding of GF to specific plasma membrane receptors. To study the constitutive expression of BCGF receptors at the B cell precursor stage of human B lineage lymphoid differentiation, the specific binding of radioiodinated HMW-BCGF and LMW-BCGF to freshly obtained B lineage lymphoblasts from B cell precursor ALL patients was investigated. Although the receptor binding assays as performed may not be sensitive enough to detect low numbers of BCGF receptors, our findings clearly indicated that leukemic B cell precursors express specific receptors for both HMW-BCGF and LMW-BCGF in a significant number of patients. By means of ^{3}H-TdR incorporation assays to measure DNA synthesis, conclusive results show that both HMW-BCGF and LMW-BCGF stimulate leukemic B cell precursors. Seventy-five percent of cases showed a significant response to LMW-BCGF, with a medium stimulation index of 9.3. By comparison, only 33% of cases showed a significant response to HMW-BCGF with a median stimulation index of 2.4. This difference may be due to expression of a higher number of surface receptors for LMW-BCGF, a higher affinity of LMW-BCGF receptors for their substrate, or both. The limited number of leukemic B cell precursors obtainable from each patient prevented extensive receptor binding assays, and a more detailed and quantitative evaluation of BCGF/BCGF receptor interactions by Scatchard analyses. Although we could not determine the exact receptor numbers, our finding are the first direct evidence of BCGF receptor expression at the B cell precursor stage of B lineage lymphoid differentiation. The numbers of cell-bound radioiodinated BCGF molecules, as well as the indices for BCGF-induced stimulation of DNA synthesis in leukemic B-cell precursors, showed a wide interpatient variation. These observations indicate the existence of a marked heterogeneity in expression of functional BCGF receptors in B cell precursor ALL. Furthermore, in many cases, the number of radiolabeled BCGF molecules bound per cell did not correlate with the response of leukemic B cell precursors to BCGF. This indicates that not all detected BCGF receptors may be functional. Alternatively, besides expression of functional receptors, the stage of activation or previous exposure to other GFs may be determining factors for BCGF response of leukemic B cell precursor cells. It is also possible that ^{3}H-TdR incorporation assays failed to measure proliferation of leukemic B cell precursors: In a recent study,[57] several components of human blood catabolized nucleosides, including thymidine, extensively. Most of the radioactivity from ^{3}H-TdR was quickly rendered unincorporable as the compound was metabolized to thymine and further breakdown products. Cells proliferated without incorporating radioactivity from the medium. Furthermore, a marked variability was found in the degree of catabolism from person to person.[57] Hence, the amount of label incorporated into the leukemic B cell precursor blasts may not reflect their actual proliferative activity.

Leukemic progenitor cells have been implicated in maintanence and expansion of leukemic cell populations; therefore, analysis of the GF requirements of this clonogenic, self-renewing subpopulation is of paramount importance. The current paucity of knowledge regarding ALL progenitor cells largely reflects the historic difficulties in cloning fresh ALL blasts in vitro.[66-70] To our knowledge, none of the purified natural or cloned GFs have been compared with respect to their biologic effects at the level of leukemic lymphoid progenitor cells. In the absence of these data, the role of GEs in the pathogenesis and biology of B cell precursor ALL is difficult to determine. We recently described a colony assay system that enables us to culture leukemic progenitor cells in B cell precursor ALL.[41] Our initial studies on B lineage leukemic lymphoid progenitor cells demonstrated a growth stimulatory role for soluble GFs provided by PHA-stimulated leukocyte-conditioned media as well as by supernatants of activated helper T cell clones.[41,56] In the present study, the proliferative responses of leukemic B lineage lymphoid progenitor cells to increasing concentrations of purified natural human HMW-BCGF and LMW-BCGF were evaluated. Among 28 cases studied, 57% responded to both HMW-BCGF and LMW-BCGF, 21% responded only to LMW-BCGF, and the remaining cases showed no proliferative response to either GF. These findings demonstrate that both HMW-BCGF and LMW-BCGF can modulate the proliferative activity of leukemic B lineage lymphoid progenitor cells. The observed increase in proliferative activity may be due to (a) stimulation of resting leukemic blasts to enter cell cycle and undergo several divisions, (b) stimulation of self-renewal of dividing blasts/shortening of cell-cycle time, or (c) both.

We observed major differences relative to the proliferative responses of the bulk of fresh leukemic marrow B cell precursors *v* their progenitor cells to HMW-BCGF. Furthermore, the nature of these differences varied between patients. These data suggest that the stage of development (ie, progenitor *v* progeny) at which HMV-BCGF exerts growth stimulatory effects may differ from patient to patient. In contrast, the proliferative response of fresh leukemic B cell precursors to LMW-BCGF correlated with the LMW-BCGF response of their progenitor blasts, indicating a rather conserved expression of LMW-BCGF receptors during early stages of B cell differentiation.

Detailed morphological, immunologic and, in a limited number of cases, cytogenetic analyses were performed to confirm the leukemic origin of colony cells in BCGF-stimulated cultures. Colony cells were TdT$^+$, surface CALLA$^+$, CD19$^+$, cytoplasmic CD22$^+$, and surface CD24$^+$, but lacked sIg or cytoplasmic μ heavy chains. These immunologic marker profiles are consistent with B cell precursor ALL. The only case with cytoplasmic μ-positive colony cells was a patient with a pre-B ALL whose uncultured bone marrow blasts expressed detectable cytoplasmic μ heavy chains. Hence, we did not observe a BCGF-induced loss of TdT, acquisition of sIg, or acquisition of cytoplasmic μ heavy chains. Although some cases were CALLA$^-$, they did express nuclear TdT, surface CD19 and cytoplasmic CD22. Based on current concepts of early B cell differentiation, we conclude that neither HMW-BCGF nor LMW-BCGFs were likely to have induced a significant differentiation of leukemic B cell precursors. Researchers have proposed that uncoupled proliferation and differentiation may represent an underlying biologic basis for neoplastic growth.[71] Remarkably, both HMW-BCGF and LMW-BCGF enhanced the proliferative activity of leukemic B cell precursors without providing detectable inductive stimuli for further differentiation.

Our immunologic marker analyses of cells growing in liquid cultures or in methylcellulose cultures provided direct evidence that (a) the increase in ^{3}H-TdR incorporation in BCGF-stimulated cultures was not due to BCGF-induced proliferation of sIg$^+$ contaminating normal B cells but rather to proliferation of leukemic B cell precursors, and (b) colonies growing in BCGF-stimulated cultures were B cell precursor colonies rather than normal sIg$^+$ B cell or T cell colonies. We then asked whether the observed increase in the proliferative activity of leukemic B lineage lymphoid progenitor cells was directly mediated by BCGFs or indirectly mediated through factors secondarily released by accessory cells. The assumption that accessory cells were involved was highly unlikely since (a) the biologic effects of both the HMW-BCGF and LMW-BCGF used in the present study are highly B lineage specific; (b) the purified BCGF preparations used in our experiments were devoid of IL 1, IL 2, GM-CSF, G-CSF, BPA, or γ-IFN; and (c) the analyzed bone marrow mononuclear cell populations were highly blast enriched. Nevertheless, virtually pure populations of FACS-sorted leukemic B cell precursors were used to formally preclude accessory cells from affecting the BCGF response of leukemic B lineage lymphoid progenitor cells. The response patterns of FACS-sorted cells to HMW-BCGFs or LMW-BCGF were essentially identical to the proliferative responses of unsorted leukemic B cell precursors. Our findings establish that both HMW-BCGF and LMW-BCGF exert direct stimulatory effects on leukemic B lineage lymphoid progenitor cells. Furthermore, this cell population represents the most immature B lineage lymphoid cells in human hematopoiesis detectable by currently available immunobiologic techniques. Therefore, the constitutive expression of functional BCGF receptors on lymphoid B lineage progenitor cells indicates that both HMW-BCGF and LMW-BCGF may have an important regulatory role for (a) early B cell development within the human B precursor pathway, as well as (b) the leukemogenesis or disease progression in B cell precursor ALL.

Interactions between hematopoietic GF have been demonstrated in in vitro systems.[1,14,17,27] Previous functional studies on HMW-BCGF and LMW-BCGF revealed that these two factors demonstrate a synergistic effect on small anti μ–stimulated B cells.[14] The present study proposed the possibility that HMW-BCGF and LMW-BCGF may functionally interact and hence have synergistic, additive, or antagonistic effects on leukemic B lineage lymphoid progenitor cells. Synergistic effects were observed in 80% of the cases that responded to both HMW-BCGF and LMW-BCGF, but were not observed in any of the cases that responded to only one form of BCGF. Our observations suggest that in some B cell precursor ALL patients, leukemic progenitor cells express distinct receptors for both HMW-BCGF and LMW-BCGF and that exposure to one form of BCGF may lower the threshold for mitogenicity of the second form of BCGF. Hence, both HMW-BCGF and LMW-BCGF may be required for maximum stimulation of these most immature B cell precursors. This hypothesis is further supported by the fact that the number of BCGF molecules binding each cell did not always correlate with BCGF response. Hence, in some cases, preexposure of leukemic B cell precursors to one form of BCGF may be a prerequisite for their subsequent response to the other form. Alternatively, two distinct nonoverlapping leukemic progenitor cell populations may be present in some patients, manifested as one clone that responds to HMW-BCGF, and another clone that responds to LMW-BCGF. This is unlikely, since synergy between HMW-BCGF and LMW-BCGF was observed only at suboptimal BCGF concentrations, and maximum stimulation of leukemic progenitor cells could be obtained with either BCGF alone at optimal concentrations. Together, these preliminary results contend that HMW-BCGF and LMW-BCGF most likely bind to different surface receptors on leukemic B cell precursors. Unambiguous identification and comparative analysis of distinct receptors for human HMW-BCGF and LMW-BCGF have not been realized, however. Future cocompetition and cross-blocking studies using radiolabeled HMW-BCGF and LMW-BCGF should elucidate the relationships/differences between BCGF receptors.

The mean maximum number of B cell precursor colonies in successful cultures showed a marked interpatient variation, ranging from 97 to 1,987 colonies/10^5 unsorted marrow blasts for HMW-BCGF, and from 14 to 2,354 colonies/10^5 unsorted marrow blasts for LMW-BCGF. These data indicate a pronounced heterogeneity in BCGF reception expression and/or BCGF response at the level of leukemic lymphoid B lineage progenitor cells in B cell precursor ALL. To comprehend this heterogeneity further, we assessed how the proliferative response of leukemic progenitor cells to HMW-BCGF or LMW-BCGF relates to more frequently measured diagnostic parameters. A very pronounced association between the BCGF response and the cytogenetic features was evident. Notably, patients with SCA involving the bands p11-13 on the short arm of chromosome 12 or patients with a Philadelphia chromosome showed a significantly greater HMW-BCGF response of leukemic progenitor cells than did patients with other types of SCA ($P < .05$). The LMW-BCGF response of leukemic progenitor cells was significantly greater for patients with SCA than for patients without SCA. Among patients with various types of SCA, there were no significant differences in the LMW-BCGF response of leukemic progenitor cells. The underlying reason for these correlations is not known. Some investigators have suggested that translocations might alter the expression of a cellular transforming gene by bringing it under the control of a new promoter/enhancer.[72,73] Accumulating evidence indicates that the products of a number of proto-oncogenes show structural and functional similarities to GF or GF receptors.[74-77] Other sets of oncogenes confer GF autonomy through indirect mechanisms, such as the postreceptor signal transduction pathways that generate a mitogenic response in a target cell after GF stimulation. The oncogenes directly implicated in these signalling pathways include the *ras* oncogene family.[78-80] C-K*ras* 2 oncogene has been mapped to 12p12 on the short arm of chromosome 12.[72] Significantly, leukemic progenitor cells from B cell precursor ALL patients with SCA involving 12p11-13 showed a marked proliferative response to both HMW-BCGF as well as LMW-BCGF. In contrast, leukemic progenitor cells from patients with SCA not involving 12p11-13 responded with much higher colony numbers to LMW-BCGF than to HMW-BCGF. These observations suggest a link between p21 *ras* oncoproteins and HMW-BCGF response, provided that in future studies patients with abnormalities of 12p11-13 differ from patients with other types of SCA in *ras* oncogene expression. Our findings are the first to demonstrate that the proliferative response of leukemic B lineage lymphoid progenitor cells to HMW-BCGF or LMW-BCGF correlates with karyotypic features. The response of leukemic B lineage lymphoid progenitor cells to HMW-BCGF or LMW-BCGF did not correlate to sex or age of patients, disease status (new diagnosis *v* relapse), FAB morphology, WBC at diagnosis, or immunophenotype.

To our knowledge, this study represents the first detailed comparative analysis of BCGF receptor expression and BCGF effects in B cell precursor ALL. The data presented provide unique and direct evidence for the expression of functional receptors for both HMW-BCGF and LMW-BCGF at the B cell precursor stage of human B lineage lymphoid differentiation. While expanding our knowledge of the biologic effects of human B cell growth factors in the early stages of human B cell development, this investigation prompts the hypothesis that HMW-BCGF and LMW-BCGF may play an important growth regulatory role in B cell precursor ALL.

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Rabbit Reticulocyte Coated Vesicles Carrying the Transferrin–Transferrin Receptor Complex: I. Purification and Partial Characterization

By Hye-Ryun Choe, Stephen T. Moseley, Jonathan Glass, and Marco-Tulio Nunez

Coated vesicles bearing the transferrin–transferrin receptor complex were isolated from rabbit reticulocytes by freeze–thaw cell lysis, followed by differential centrifugation with pelleting of vesicles at 100,000 g. Electronmicroscopy demonstrated the vesicles to have the characteristic morphology of coated vesicles, including the appearance of triskelions. The protein composition of the vesicles as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis included transferrin, transferrin receptor, and proteins of apparent mol wt of ~180,000, 140,000, 100,000, and 47,000 daltons. The 180,000 and 100,000 mol wt proteins were identified as clathrin and coated vesicle assembly factor proteins, respectively, by Western blot analyses. The vesicles had a Mg^{2+}-dependent ATPase with a specific activity of ~8.5 nmoles ATP converted/min/mg vesicle protein. The vesicles could acidify the intravesicular space, as evidenced by the stimulation of the Mg^{2+}-ATPase by the protonophore FCCP. Reticulocytes appear to be an excellent source of coated vesicles and as such should provide a model for studying the endocytosis of transferrin and the steps of iron uptake that procede in these vesicles.

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AMPLE EVIDENCE from studies both with reticulocytes and cultured cells shows that transferrin, after binding to its specific receptor on the cell surface, is internalized into a compartment in which release of iron from transferrin occurs. Fluorescent or electron-dense markers and ultrastructural studies have been used to demonstrate that transferrin enters cells through coated vesicles.[1-6] In reticulocytes, part of the initial coated vesicle population may form multivesicular bodies that then undergo exocytosis.[3] In this process, transferrin appears to be located within a vesicular compartment, since bound transferrin is resistant to exchange from the cell[7] and to digestion with pronase.[8-10] Many of these same studies, as well as others, demonstrate that the compartment into which transferrin is internalized is acidified.[2,11,12] The acidic milieu is essential both for the release of iron from transferrin[13] and for the continued binding of apo-transferrin to the transferrin receptor, allowing the apo-transferrin to cycle out of the cell undegraded.[14] Recent studies from our laboratory[15,16] demonstrate that the role of acidification may be more complex, with an acid intravesicular pH required for both dissociation of iron from transferrin and for proper transport of released iron through the vesicle membrane. During the process of translocation of released iron through the vesicle membrane, iron is reduced from Fe (III) to Fe (II).[17] A model based on these various studies suggests that the internalized compartment must have both a proton pump driven by an ATPase capable of acidifying the vesicle and a reductase capable of reducing iron.[15] In the following studies, we isolated from rabbit reticulocytes clathrin-coated vesicles bearing the transferrin–transferrin receptor complex, characterized the unique protein composition of these vesicles, and defined a Mg^{2+}-dependent ATPase that can acidify the intravesicular space.

MATERIALS AND METHODS

Vesicle preparation. Rabbit reticulocytes, derived at least 4 days after a course of phenylhydrazine,[15] were depleted of bound transferrin by two 10-minute incubations at 37°C in phosphate-buffered saline[1] (PBS) supplemented with 5 mmol/L glucose and 0.2% bovine serum albumin (BSA). The cells were then incubated for 8 minutes at 37°C with rabbit diferric ^{59}Fe, ^{125}I-transferrin prepared as previously described,[17] and the labeled cells were then incubated at 4°C in PBS-5% rabbit serum to exchange labeled transferrin that was bound but not internalized. The cells were suspended in 1 vol lysis buffer consisting of 100 mmol/L 2-[N-Morpholino] ethanesulfonic acid (MES) (pH 6.5), 1 mmol/L EGTA, 0.5 mmol/L $MgCl_2$, 0.2 mmol/L phenylmethyl sulfonyl fluoride (PMSF) and lysed by two cycles of rapid freezing in dry ice-acetone followed by thawing by continuous swirling in an ice bath. The lysed cells were pelleted at 9,500 rpm for 10 minutes in a Sorvall SS-34 rotor, and the supernatant (S_1) was centrifuged in a Beckman SW 27 rotor at 25,000 rpm for 60 minutes. The resulting pellet (P_2) was resuspended in lysis buffer by brief sonication in a bath-type sonicator, nonsuspended material was pelleted at 9,500 rpm for 10 minutes, and the supernatant (S_3) was pelleted again at 25,000 rpm to yield the final pellet (P_4). In addition, plasma membranes and mitochondria were prepared from rabbit reticulocytes as described.[18]

Analytic studies of vesicle preparations. P_4 was examined by electronmicroscopy both by negative staining with 2% uranyl acetate and rotary-replication with platinum after being sprayed onto mica in 50% glycerol as previously described.[19] In some experiments, S_3 was further fractionated on isopycnic density gradients of 30% Percoll (Pharmacia, Piscataway, NJ) centrifuged at 15,000 rpm for 45 minutes, with the gradients subsequently harvested from the bottom of the tube. Protein composition of the vesicles was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 7.5% acrylamide using Laemmli buffer systems.[20] Vesicle

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proteins separated by PAGE were transferred to nitrocellulose paper for Western blotting. The presence of clathrin and the ~100,000 dalton clathrin assembly factor protein was assayed with rabbit anti-clathrin or 100,000-dalton protein antibodies using a horseradish peroxidase-coupled goat anti-rabbit IgG as the second antibody. Characterization of these antibodies against coated vesicle proteins will be described in detail elsewhere (S.T. Mosley, and D. Branton, manuscript in preparation).

Measurement of ATPase activity. The ATPase activity was assayed by monitoring NADH oxidation in a modification of the coupled assay system described by Cantley and Josephson.[21] Vesicle preparations were resuspended in 100 mmol/L MES (pH 6.5), 1 mmol/L EGTA to a protein concentration of 0.5 to 1.0 mg/mL, and the reaction was started by the addition of aliquots of 30 to 100 μL to the same MES–EGTA buffer containing 1.5 mmol/L phosphoenolpyruvate (Sigma, St Louis), 7 to 10 U/mL of a mixture of pyruvate kinase and lactic dehydrogenase (Sigma), 3 mmol/L ATP, 8 mmol/L $MgCl_2$, and 0.1 mg/mL NADH. The change in absorbance at 338 nm was monitored in a dual-beam spectrophotometer using the reaction mixture without vesicles in the reference cuvette. The effect of the inhibitors ouabain, oligomycin, NaN_3, NBD-Cl, and vanadate were studied at 1 mmol/L, 0.5 mmol/L, 2 mmol/L, 10 μmol/L, and 10 μmol/L, respectively, after preincubation with vesicles for 1 to 2 hours at room temperature. The effect of the protonophore FCCP was assayed by addition to a final concentration of 5 μg/mL to the cuvette during the reaction.

RESULTS

Electronmicroscope characterization of vesicles isolated from reticulocytes. As shown in Fig 1, vesicles were visualized both by negative staining with uranyl acetate and rotary-shadowing electronmicroscopy. The predominant material (Fig 1A) was clathrin-coated vesicles, which constituted almost all of the material in P_4 and appeared similar to brain-coated vesicles (not shown). By rotary-shadowing (Fig 1B), the characteristic triskelions, released from coated vesicles during spraying in glycerol, were found. This confirmed the identity of the isolated material in P_4 as clathrin-coated vesicles.

Protein composition and characterization of the vesicles. The yield of vesicles obtained after freeze-thaw lysis was ~1 mg of vesicle protein from 10 mL packed RBCs with a reticulocyte count of ~50%. This yield compares favorably with the yields obtained both with bovine brains[22] and hepatocytes.[23] The density of the vesicles as measured by Percoll density gradient centrifugation was 1.043 g/cm^3, similar to the density of coated vesicles isolated from other sources. The uniqueness of the protein composition of the vesicles was demonstrated by examining the vesicles by SDS-PAGE. As shown in Fig 2A, lane 3, the protein composition of the reticulocyte-coated vesicles fraction is clearly distinct and markedly different from reticulocyte plasma membrane. Six proteins, either not discernible, or barely so, in the plasma membrane but quite prominent in the vesicles are transferrin, transferrin receptor, and proteins of apparent mol wt 180,000, 140,000, 100,000, and 47,000. In contrast, erythrocyte plasma membrane bands 1, 2, 2.1, and 3 are depleted from the vesicle preparations. Further purification of vesicles by gel filtration chromatography through Biogel A1.5 did not change the protein pattern on subsequent analysis by SDS-PAGE or change the relative amounts of the major proteins, suggesting that contamination by other cellular structures, if any, is negligible. The yield and enrichment of transferrin (and hence the transferrin receptor) during purification of vesicles was assessed by determining the recovery of ^{125}I-transferrin bound to reticulocytes. As shown in Table 1, an enrichment of transferrin of ~1,000-fold was achieved between the freeze–thaw lysate, S_1, and the coated vesicles in P_4.

In addition to the data from electronmicroscopy, three lines of evidence suggest that these are clathrin-coated vesicles: (a) The 180,000-dalton polypeptide was released from the vesicles on treatment with 10 mmol/L Tris, pH 8.5 (Fig 2A, lane 4), as has been described for the release of clathrin from coated vesicles derived from brain.[24] In addition, the proteins of mol wt of ~140,000 and 100,000 daltons were also released under these conditions. (b) Western blot analysis with anti-clathrin polyclonal antibody showed binding to a single polypeptide of 180,000 daltons (Fig 3). (c) Antibody against the ~100,000-dalton polypeptide associated with coated vesicle bound to a polypeptide of similar mol wt in the reticulocyte vesicle preparation (Fig 3).

Sidedness of the vesicles. Evidence that transferrin was contained within the vesicles was obtained from the ability to

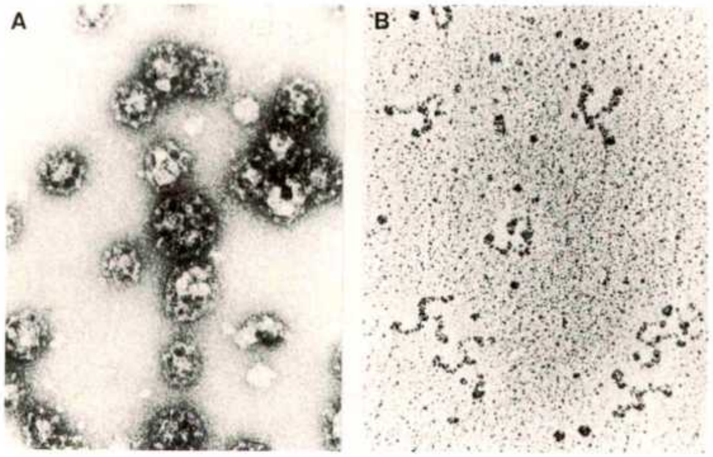

Fig 1. Electronmicrograph of reticulocyte clathrin-coated vesicles (fraction P_4). (A) Coated vesicles were negatively stained with uranyl acetate and range in size from 90 to 120 nm. (Original magnification ×77,000; current magnification ×30,800.) (B) Platinum rotary replication of the reticulocyte coated vesicles show the three-legged triskelion shape characteristic of clathrin heavy chains. (Original magnification ×149,000; current magnification ×59,600.)

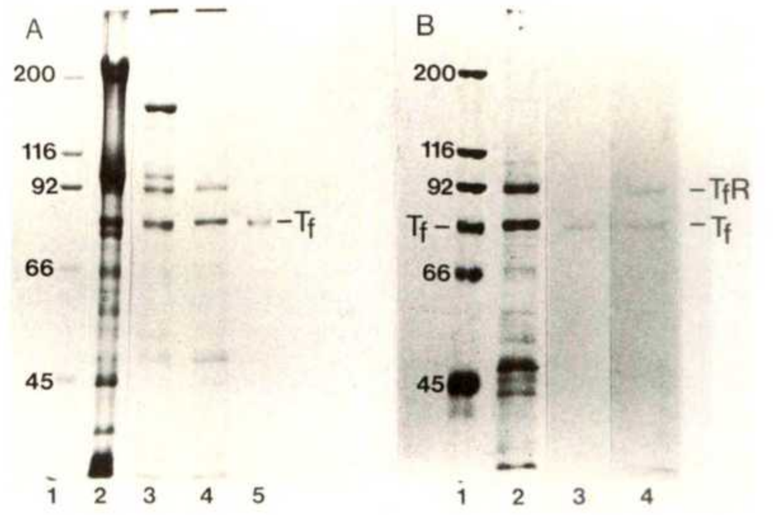

Fig 2. SDS-PAGE of vesicle preparations and identification of transferrin (Tf) and transferrin receptor (TfR) by Western blot analyses. Vesicles were prepared and electrophoresed as described in the Materials and Methods Section, and the gel was stained with Coomassie blue (A) or transferred onto a nitrocellulose paper to identify Tf and TfR, using anti-Tf and TfR antibodies, respectively (B). (A) Lane 1 represents mol wt markers; lane 2, reticulocyte plasma membrane; lane 3, coated vesicle preparation (P_4); lane 4, clathrin-depleted vesicles in 10 mmol/L Tris, pH 8.0; and lane 5 represents purified rabbit Tf. (B) Lanes 1 and 2 are Coomassie blue staining of mol wt markers and Tf (lane 1) and clathrin-depleted vesicles (lane 2); lanes 3 and 4 are Western Blot analyses with goat anti-rabbit Tf antibody (lane 3) and goat anti-rabbit Tf receptor antibody (lane 4) using horseradish peroxidase-conjugated rabbit anti-goat IgG as a second antibody. Cross-reaction of Tf receptor antibody with Tf in lane 4 is due to the Tf contamination in Tf receptor preparation used for immunization. Molecular masses of the marker polypeptides given figure are in kilodaltons.

exchange at 0°C <5% of bound ^{125}I-transferrin with unlabeled excess transferrin. The asymmetrical distribution of the proteins could also be demonstrated by ^{125}I-labeling of the proteins of vesicles prepared with unlabeled transferrin. Iodination with ^{125}I by lactoperoxidase to label the externally facing vesicle proteins, followed by SDS-PAGE and radioautography demonstrated that the clathrin as well as the transferrin receptor, a known transmembrane protein, were labeled while transferrin itself was not (data not shown).

ATPase activity and acidification of the vesicles. The ATPase activity of the vesicles was assayed by monitoring NADH oxidation at 338 nm.[21] The ATPase activity of the vesicles was Mg^{2+} dependent and had an average specific activity of 8.5 nmoles ATP converted/min/mg vesicle protein at room temperature. As shown in Table 2, the ATPase activity of the vesicles could be distinguished from the ATPase activity of plasma membrane and mitochondria by the pattern of inhibition with ouabain, oligomycin, NBD-Cl, sodium azide, and vanadate. The vesicle ATPase activity was not inhibited by ouabain, minimally inhibited by oligomycin, vanadate and sodium azide, and inhibited ~40% by NBD-Cl. FCCP, an electrogenic protonophore,[25] stimulated the ATPase activity, suggesting that the ATPase was acidifying the interior of the vesicle. This hypothesis has been tentatively confirmed by determining the ATP-dependent alkalinization of the extravesicular medium using a pH-sensitive electrode and quenching of acridine orange fluorescence. In contrast to the above, there was a lack of effect of FCCP on ATPase activities of plasma membrane and mitochondria.

DISCUSSION

The reticulocyte is uniquely suited for isolation of the internalized compartment containing the transferrin–transferrin receptor complex: Transferrin receptors are the predominant cell surface receptor; internal membrane structures are reduced in reticulocytes as compared with other cells; and, as shown in the present studies, a high yield of clathrin-coated vesicles may be obtained. The reticulocytes were disrupted under mild hypotonic conditions by freeze–thaw lysis to preserve the integrity of the vesicles. Low-speed centrifugation was sufficient to remove unbroken cells and

Table 1. Purification of Coated Vesicle-Associated ^{125}I-Transferrin During Vesicle Preparation

Fraction	Transferrin (molecules/mL RBC^{-1} × 10^{13})	Specific Activity (molecules Tf/mg protein^{-1} × 10^{13})	Yield (%)	Purification (– fold)
S_1	17.09 ± 3.68	0.017 ± 0.0047	100	1
P_2	6.96 ± 3.56	3.03 ± 2.50	31.40 ± 6.90	174
P_4	4.47 ± 1.84	19.77 ± 3.90	25.30 ± 9.20	1,136

Vesicles were prepared as described in the Materials and Methods section from reticulocytes incubated with ^{59}Fe, ^{125}I-transferrin. Radioactivity and protein content were determined at each of the indicated steps of purification. The results are the means ± SD of five experiments with an average reticulocyte count of 45%.

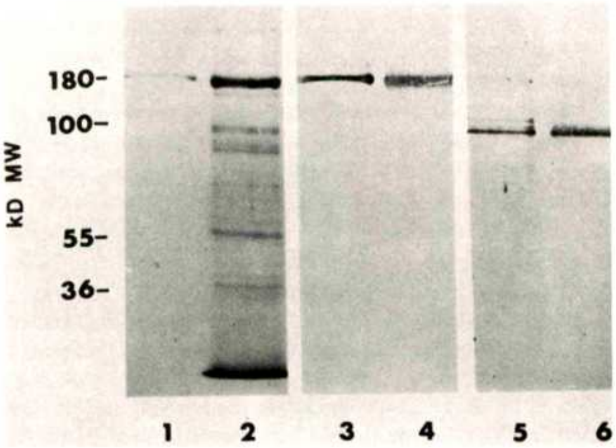

Fig 3. Western blot of SDS-PAGE-fractionated bovine brain coated vesicles (lanes 1, 3, and 5) and reticulocyte coated vesicles (lanes 2, 4, and 6). Lanes 1 and 2 show total protein by Ponceau S stain; lanes 3 and 4 are blotted with anti-clathrin antiserum; lanes 5 and 6 are blotted with anti-100,000 dalton assembly factor antiserum. Antisera were diluted 1:1,000 in PBS/5% newborn calf serum/0.1% Tween 20 for incubation with nitrocellulose strips.

Table 2. The Mg^{2+}-Dependent ATPase Activity of Vesicles, Plasma Membrane, and Mitochondria

	ATPase Activity (% Control)					
Fraction	Ou	Olig	NBD	Van	NaN_3	FCCP
Vesicle	100	94	61	90	84	145
Plasma membrane	44	67	69	61	92	108
Mitochondria	96	45	31	103	15	89

Mg^{2+}-dependent ATPase activity of rabbit reticulocyte vesicles, plasma membrane, and mitochondria were measured as described in the Materials and Methods section in the absence and presence of 1 mmol/L oubain (Ou), 0.5 mmol/L oligomycin (Olig), 10 mol/L NBD-Cl (NBD), 10 μmol/L vanadate (Van), 2 mmol/L sodium azide (NaN_3) and 5 μg/mL FCCP. The results, the ATPase activity expressed as percentage of treated sample of the untreated control, are the means of three experiments.

mitochondria, whereas the subsequent ultracentrifugation removed plasma membrane fragments. The resulting vesicle preparations had a characteristic protein composition. The major vesicle protein had an apparent mol wt by SDS-PAGE of 180,000 daltons, and our studies with anti-clathrin antibody identified the protein as clathrin. This protein, forming the characteristic triskelions on the vesicles, could be removed from the vesicles under alkaline conditions as described for other coated vesicles.[24] These studies confirm a recent report on the presence of clathrin in mature erythrocytes, with the hypothesis that erythrocyte clathrin reflects residual clathrin from endocytic vesicles in immature cells,[27] and a report on the presence of coated pits in reticulocytes.[28] The uniqueness of the protein composition of the reticulocyte-coated vesicles was further substantiated by finding that cytoskeletal proteins were minimally present, consistent with the observations that the invaginating domains of membrane in the process of endocytosis are devoid of spectrin.[29,30] The major transmembrane protein, band 3, was not detected in the vesicles, whereas transferrin and the transferrin receptor, which are barely detectable on electrophoresis of reticulocyte plasma membranes, were prominent constituents of the vesicles. Furthermore, the purification of transferrin (and hence the transferrin receptor) was considerable, suggesting that a unique array of proteins was isolated in the vesicles. Dickson et al[31] have also demonstrated enrichment of transferrin receptors in endosomes from a human cell line.

Acidification of the compartment into which transferrin is internalized is essential for iron uptake in reticulocytes.[2,11,16] Presumably, reticulocytes lower the pH of the endocytic vesicles by an ATP-dependent proton transport similar to that described for coated vesicles derived either from brain or liver.[32,33] The ATPase activity of the vesicles could be demonstrated by response to cations and various inhibitors to be similar to that of clathrin-coated vesicles derived from calf brain[32,33] and distinct from those of reticulocyte plasma membrane or mitochondria. The lack of inhibition of vesicle ATPase activity by ouabain and sodium azide distinguishes this ATPase from ATPases in plasma membrane or mitochondria. In contrast to the findings with brain coated vesicles,[33,34] inhibition of erythrocyte ATPase activity by NBD-Cl was not nearly as potent or as specific for coated vesicles. The ability to stimulate vesicle ATPase activity with the protonophore FCCP provided indirect evidence that the Mg^{2+}-ATPase was the driving force for proton accumulation within the vesicle. The extent of stimulation by FCCP was similar to that previously observed with brain coated vesicles.[33]

These vesicle preparations provide a relatively rapid method for isolating large quantities of coated vesicles bearing transferrin bound to the transferrin receptor. As such, these vesicles provide a model for studying several of the components essential for iron uptake, including an ATP-driven proton pump, a membrane iron carrier, and a reductase to reduce Fe (III) to Fe(II) for transport into the cytosol. Because of the relative simplicity of the reticulocyte system, the ease of obtaining reticulocytes, and the extent of knowledge of the RBC membrane proteins, the reticulocyte vesicles may also provide a model for studying the early events of receptor-mediated endocytosis, including the segregation of unique proteins into the coated pit, the process of vesiculation of the coated pit, and the passage of receptor-ligand complexes from coated vesicles into endosomes.

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Platelet-Associated and Plasma Anti-Glycoprotein Autoantibodies in Chronic ITP

By R. McMillan, P. Tani, F. Millard, P. Berchtold, L. Renshaw, and V.L. Woods, Jr

Chronic immune thrombocytopenic purpura (ITP) is due to platelet destruction by circulating antiplatelet antibody. Although autoantibodies against the platelet glycoprotein IIb/IIIa (GPIIb/IIIa) complex and GPIb have been demonstrated using various methods, practical assays for detection of platelet-associated or plasma autoantibodies have not been available. We studied 59 patients with chronic immune thrombocytopenic purpura in whom platelet-associated and plasma autoantibodies against the GPIIb/IIIa complex and GPIb were measured using a newly developed immunobead assay and a previously reported microtiter-well assay. Platelet-associated autoantibody was detected using the immunobead assay in 21 of 28 patients (75.0%; 13 with anti-GPIIb/IIIa, 8 with anti-GPIb). Plasma autoantibodies were noted in 34 of 59 patients (57.6%; 21 with anti-GPIIb/IIIa, 11 with anti-GPIb, and 2 with both). Positive results were noted in 30 of 59 patients using the immunobead assay and in only 14 of 59 using the microtiter-well assay, suggesting that solubilization of the platelets prior to antibody addition, as in the microtiter-well assay, alters epitope stability. Of the 31 thrombocytopenic control patients studied, all gave negative results using both assays. We conclude that these clinically adaptable assays allow detection of autoantibodies in most patients with chronic ITP, confirming the presence of an autoimmune process.

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CHRONIC IMMUNE thrombocytopenic purpura (ITP) is a syndrome of destructive thrombocytopenia due to an antibody against a platelet-associated antigen.[1,2] Van Leeuwen et al[3] provided the first evidence for autoantibodies in ITP patients when they noted that 32 of 42 antibody eluates from ITP platelets would bind to normal but not to thrombasthenic platelets. Because thrombasthenic platelets are deficient in platelet glycoproteins (GP) IIb and IIIa, they suggested that these ITP patients had autoantibodies to one of these GPs. Direct evidence for anti-GP autoantibodies in chronic ITP has been provided by subsequent studies. Woods et al showed binding of autoantibodies from ITP patients to the GPIIb/IIIa complex or to GPIb attached to microtiter wells with monoclonal antibodies and confirmed these observations by immunoprecipitation.[4,5] They noted anti-GPIIb/IIIa or anti-GPIb autoantibodies in ~10% of patients, much less than the percentage observed by the indirect studies of van Leeuwen et al.[3] Other investigators also detected autoantibodies in chronic ITP patients using immunoblotting,[6,7] immunoprecipitation,[4,5,8] inhibition of murine monoclonal anti-GPIIb/IIIa antibody binding to ITP platelets,[9] and crossed immunoelectrophoresis.[10] Nugent et al[11] and Asano et al[12] established human hybridomas from ITP lymphocytes that synthesize monoclonal antiplatelet antibodies, some of which are specific for platelet GPs.[11]

Of the assays used for demonstrating anti-GP autoantibodies in chronic ITP, the microtiter-well assay[4,5] is most easily adaptable to clinical use. The low percentage of positive tests (~10%), however, when compared with that of van Leeuwen et al (~76%) suggested to us that solubilization of the platelets prior to antibody sensitization may alter some of the epitopes. For this reason, we designed an assay (immunobead assay) for anti-GP autoantibodies where platelets are sensitized prior to their solubilization, postulating that epitopes may remain more stable when bound to antibody. This assay can measure both platelet-associated and plasma autoantibodies We studied 59 chronic ITP patients and noted platelet-associated autoantibodies in 21 of the 28 patients and plasma autoantibodies in 34 of 59 patients.

MATERIALS AND METHODS

Study subjects. We studied plasma or platelet samples obtained between January 1, 1982, and September 1, 1986, from 59 patients with chronic ITP, 20 control subjects, and 31 thrombocytopenic controls [acute nonlymphocytic leukemia, 5 patients; acute lymphoblastic leukemia, 2 patients; bone marrow transplantation patients with (4 patients) and without (2 patients) anti-HLA antibodies; aplastic anemia, 2 patients, non-Hodgkins lymphoma, 4 patients (one with cryoglobulins); cirrhosis, 1 patient; myeloproliferative disease, 4 patients; carcinoma on chemotherapy, 3 patients; systemic lupus erythematosus with thrombocytopenia, 3 patients; and immune neutropenia, 1 patient].

Patients with ITP were thrombocytopenic with normal or increased numbers of megakaryocytes and without evidence of other types of immune thrombocytopenia. Platelet-associated and plasma autoantibodies were both studied in 28 of 30 patients seen after March 1986 when the immunobead assay first became available; in 2 of these patients, we could not obtain sufficient platelets for study. Prior to March 1986, only plasma samples were available for assay. The majority of patients (46 of 59) were seen and studied at our institution; the remainder were seen by physicians in the San Diego area (5 patients) or in other parts of the country (8 patients), and samples were referred to us for study The patient group is biased toward patients with more severe disease as reflected by the large number (24 patients) who were first studied after having failed to attain a remission with splenectomy.

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In all studies in this report, EDTA-anticoagulated blood was studied. Preliminary studies have shown that similar results can be obtained with either ACD-A–anticoagulated blood or serums although serum tends to give higher background values in the microtiter well assay.

Assays for anti-GP autoantibody The specificity of both the immunobead and microtiter assays is determined by the monoclonal anti-GP antibody used. Both assays are shown schematically in Fig 1. The following murine monoclonal antibodies were used: anti-GPIIb/IIIa-2A9, 3F5, 2G12—2A9 is specific for GPIIb, and the others are complex-specific; anti-GPIb-P3 (provided by Drs Zaverio Ruggeri and Theodore Zimmerman, Scripps Clinic); and anti-human IgG (American Type Culture Collection, Rockville, MD, ATCC HB-43). Monoclonal antibodies (50 μg) were labeled with 500 μCi of ^{125}I using the chloramine-T method. All incubations in both assays were carried out at room temperature.

In detergent extracts GPIIb and GPIIIa are known to form a complex and GPIb is known to be complexed with platelet GP IX. Therefore, as measured by these two assays, anti-GPIIb/IIIa autoantibodies could be against epitopes on either GPIIb or GPIIIa and anti-GPIb specific for GPIb or GP IX. For the purposes of this report, however, results are reported as either anti-GPIIb/IIIa or anti-GPIb, referring to autoantibodies against proteins of the GPIIb/IIIa complex or the GPIb complex.

Immunobead assay. The immunobead assay can be used to measure either platelet-associated autoantibody or plasma autoantibody.

Immunobead preparation. Anti-IgG–coated immunobeads were prepared by incubating polystyrene beads (Poly-Sep, Polysciences, Warrington, PA) with murine monoclonal anti-human IgG (ATCC HB-43) in 0.1M NaHCO$_3$, pH 8.5 for 60 minutes at a bead/antibody ratio of 2,000:1 by weight (eg, beads 100 mg/50 μg of anti-IgG in 2 mL saline). The beads were then centrifuged for 10 seconds at maximum buffer speed in a tabletop centrifuge (International Clinical Centrifuge, Boston, Mass, Model 54133M). After being washed once with 10 mL 0.05% Tween-20 in phosphate-buffered saline, pH 7.4 (PBS-Tween), nonspecific binding sites were blocked by incubation of the beads in 2% bovine serum albumin (BSA) in PBS-Tween for 60 minutes, followed by four washes in PBS-Tween. For recent assay improvements, see "note added in proof" at the end of the manuscript.

Platelet preparation Platelets from EDTA-anticoagulated blood were obtained from the patient or from a normal donor and washed six times with 0.05 mol/L isotonic citrate buffer.[13] For preparation of antibody-sensitized platelets, washed normal platelets (10^8 in 0.1 mL) were incubated with 900 μL of patient or control plasma, containing PGE$_1$ (1 μg/mL) and theophylline (1 μmol/L), for 60 minutes at room temperature and then washed four times with 0.05 mol/L citrate buffer containing PGE$_1$ and theophylline. Patient platelets (10^8) or the antibody-sensitized platelets were resuspended in 900 μL citrate buffer containing leupeptin (100 μg/mL) and then solubilized by the addition of 100 μL of 10% Triton X-100. Control samples were handled similarly.

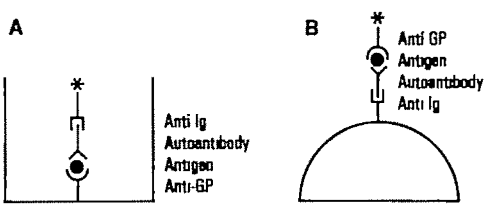

Fig 1. Antiplatelet autoantibody assays. (A) Microtiter-well assay; (B) immunobead assay.

Assay The solubilized platelets from each sample were centrifuged at 12,000 g for 5 minutes. Preliminary studies showed that this step was required, particularly in anti-GPIb autoantibody studies, to prevent falsely elevated values. Nine hundred microliters of supernate was then incubated for 60 minutes with 100 mg of anti-IgG–coated immunobeads to allow attachment of IgG and any bound antigen. After four washes with PBS-Tween, the presence of specific antigen was demonstrated by incubating the beads with 1.0 mL PBS-Tween containing ~400,000 cpm of ^{125}I monoclonal antibody specific for either anti-GPIIb/IIIa (a cocktail of three monoclonal antibodies specific for noncompeting sites, 2A9, 3F5, and 2G12) or anti-GPIb (P$_3$) for 60 minutes at room temperature and then washing them four times with PBS-Tween. The beads were resuspended in 1 mL buffer, and 0.5 mL was removed for determining radioactivity. Data are expressed as a binding ratio of cpm of patient sample/mean cpm of three control samples. The mean percentage of variation results of replicate samples of control platelets and platelets sensitized with control plasma were anti-GPIIb/IIIa- 10.1 ± 7.5 (14 studies) and 9.1 ± 8.1 (31 studies), respectively, and anti-GPIb- 6.8 ± 7.0 (17 studies) and 7.9 ± 7.6 (24 studies), respectively. Patient samples with a binding ratio of >1.3 are considered positive (>2 SD over control). The radioactivity bound to control beads ranged from 500 to 1,200 cpm depending on the activity of the labeled monoclonal antibody and the time from labeling to its use. Antibodies were freshly labeled at least every 4 weeks. Preliminary studies show that positive reactivity can be removed by adsorption of plasma with excess platelets. Storage of samples for up to 4 days at 4°C did not affect platelet or plasma control values.

Microtiter-well assay. Details have been previously published.[4,5] In brief, washed platelets (10^9 mL) or CEM leukemic cells (10^7 mL) in PBS containing leupeptin (100 μg/mL) were solubilized in 1% Triton X-100 for 30 minutes at 4°C and then ultracentrifuged (100,000 g for 60 minutes). The lysates were stored at -70°C. Microtiter wells were coated overnight at 5°C with 100 μL of either anti-GPIIb/IIIa (2A9 or 3F5) or anti-GPIb (P3) at a concentration of 5 μg/mL. After six washes with 200 μL PBS-Tween, the remaining binding sites were blocked for 60 minutes with 200 μL 2% BSA in PBS-Tween. After six washes with PBS-Tween, 100 μL of platelet lysate or the antigen negative CEM lysate, diluted 1:10, was added and incubated for 60 minutes. This allows attachment of the specific platelet antigen to the well-bound monoclonal antibody. After six washes, appropriate dilutions (1:10 for screening plasma and higher dilutions if positive) of patient or control plasma were added and incubated for 60 minutes. After six washes, 100 μL of radiolabeled murine monoclonal antihuman IgG (~100,000 cpm) was added and, after 60-min incubation and six final washes, the radioactivity of each well was determined. The percentage of variation for replicate control plasmas is -5.0 ± 7.7 for anti-GPIIb/IIIa and -1.9 ± 6.4 for anti-GPIb.[4,5] Samples with a percentage of increase of >11 were considered positive (>2 SD).

RESULTS

Chronic ITP patients. The results in all patients are summarized in Table 1. The data on individual patients are also analyzed in terms of autoantibody patterns: group 1, 10 patients who were positive in both the immunobead (platelet-associated or plasma antibodies) and microtiter-well assays (Table 2); group 2, 26 patients who were positive in the immunobead assay (platelet-associated or plasma antibodies) but negative in the microtiter-well assay (Table 3); group 3, 4 patients who were positive in the microtiter-well assay but negative in the immunobead assay (Table 4); and

Table 1. Summary of Antiplatelet Glycoprotein Autoantibody Results

	Entire Group	Assay Pattern Group* 1	2	3	4
No. of patients	59	10	26	4	19
Platelet-associated antibody					
Total no studied	28	5	16	1	6
No. positive	21 (75.0%)	5	16	0	0
Anti-GPIIb/IIIa	13	2	11	—	—
Anti-GPIb	8	3	5	—	—
Plasma antibody					
Total no studied	59	10	26	4	19
No. positive	34 (57.6%)	10	20	4	0
Anti-GPIIb/IIIa	21	4	16	1	—
Anti-GPIb	11	5	4	2	—
Both	2	1	0	1	—
Splenectomy results					
No of surgeries	44	8	20	3	13
Complete remissions	12	0	6	1	5

*Group 1, positive in both assays; group 2, positive in only the immunobead assay, group 3, positive in only the microtiter-well assay; and group 4, negative in both assays.

group 4, 20 patients who were negative in both assays (Table 5). It was not possible to correlate the degree of autoantibody positivity with the severity of the patient's disease, since samples were obtained at different times during the patient's disease and when they were receiving different types of treatment.

Platelet-associated autoantibody. Platelet-associated autoantibody was measured using the immunobead assay in 28 patients (Tables 2 and 3). Of these, 21 (75.0%) were positive, with ratios ranging from 84.5 to 2.2 (control values <1.3); 19 of the 21 patients had ratios of ≥5.0, with a mean ratio of 18.6. Thirteen patients had anti-GPIIb/IIIa, and eight had anti-GPIb autoantibodies. In six patients, only platelet-associated autoantibodies were detected and the patients had negative plasma assays.

Platelet-associated autoantibodies in three patients (patients 24, 26, and 29) were studied before and after splenectomy. In the patient who attained a complete remission (patient 29), the anti-GPIIb/IIIa autoantibodies were no longer present after splenectomy. In the two others, the autoantibodies persisted despite splenectomy and although both obtained a temporary increase in the platelet count after surgery (135,000/μL in patient 24 and 313,000/μL in patient 26), they both became severely thrombocytopenic (<20,000) within a few weeks after surgery.

Plasma autoantibody. Plasma autoantibodies were studied in 59 patients using both the immunobead (58 patients) and microtiter-well assay (59 patients). Thirty-four patients (57.6%) were positive; 10 were positive against the same antigen with both assays (Table 2), and 20 were positive with the immunobead assay alone (Table 3). Four samples were positive with the microtiter-well assay alone (Table 4). Of the 34 positive samples, 21 patients had anti-GPIIb/IIIa autoantibodies, 11 had anti-GPIb autoantibodies, and 2 had both types (patient 6 and patient 32).

Autoantibody results and splenectomy. Because many of our patients were studied for the first time after failing splenectomy (24 patients), we believed that these results might not reflect the true incidence of positive tests in newly diagnosed patients. For this reason, we compared the results obtained from patients studied initially prior to surgery with those from patients studied for the first time after failing splenectomy. Neither the number of positive platelet-associated autoantibodies (12 of 17 preoperatively; 9 of 11 postoperatively) nor the number of positive plasma autoantibodies (19 of 32 preoperatively; 15 of 21 postoperatively) differed significantly between the two groups. The number of patients testing positive for both assays was much greater in those studied first after surgery (8 of 15 postoperatively; 1 of 17 preoperatively), whereas those positive in the immunobead assay alone were the reverse (14 of 17 preoperatively; 5 of 15 postoperatively).

Thrombocytopenic controls Platelets (15 patients) and plasma (20 patients) from 31 patients with nonimmune thrombocytopenia were studied. Negative results were obtained in every instance using the immunobead and the microtiter assays (data not shown).

Table 2. Antiplatelet Glycoprotein Autoantibodies: Group 1, Positive With Both Assays

				Anti-GP IIb/IIIa			Anti-GPIb		
				Immunobead			Immunobead		
Patient	Platelet Count/μL	SPLX	Resp	P-Assoc	Plasma	Well	P-Assoc	Plasma	Well
1	6,000	Yes	No	7.0*	9.4	Pos (640)	—	Neg	Neg
2	7,000	Yes	No	—	5.2	Pos (320)	—	Neg	Neg
3	11,000	Yes	No	84.5	28.5	Pos (80)	Neg	Neg	Neg
4	117,000	Yes	No	—	2.1	Pos (40)	—	Neg	Neg
5	1,000	Yes	No	—	—	Neg	—	3.3†	Pos (6,400)
6	3,000	Yes	No	—	Neg	Pos (40)	—	2.6	Pos (80)
7	7,000	Yes	No	—	Neg	Neg	17.2	2.0	Pos (20)
8	14,000	Yes	No	Neg	Neg	Neg	5.7	1.3	Pos (80)
9	15,000	No	—	Neg	Neg	Neg	43.1	19.4	Pos (40)
10	112,000	No	—	—	Neg	Neg	—	3.6	Pos (40)

Abbreviations: SPLX, splenectomy; Resp, response, Yes, normalization of platelet count; No, no response or relapse, Immunobead, results of platelet-associated (P-Assoc) or plasma immunobead assay expressed as a ratio of patient/control results (positive >1.3); Well, results of microtiter-well assay expressed as negative (neg) or positive (pos) with titers of positive tests shown in parentheses.

*Plasma (0.1 mL) used to sensitize platelets.

†Platelets (0.18 × 10^8) assayed.

Table 3. Antiplatelet Glycoprotein Autoantibodies: Group 2, Positive Immunobead Assay/Negative Microtiter-Well Assay

				Anti-GP IIb/IIIa			Anti-GPIb		
				Immunobead			Immunobead		
Patient	Platelet Count/μL	SPLX	Resp	P-Assoc	Plasma	Well	P-Assoc	Plasma	Well
11	2,000	Yes	Yes	16.7	2.3	Neg	—	Neg	Neg
12	3,000	Yes	No	—	2.2	Neg	—	Neg	Neg
13	4,000	Yes	No	6.3	2.0	Neg	Neg	Neg	Neg
14	4,000	Yes	No	5.0	Neg	Neg	Neg	Neg	Neg
15	9,000	Yes	No	—	4 5	Neg	—	Neg	Neg
16	13,000	Yes	No	—	2.5	Neg	—	Neg	Neg
17	14,000	Yes	No	—	1.5	Neg	—	Neg	Neg
18	19,000	No	—	—	1.4	Neg	—	Neg	Neg
19	21,000	Yes	No	—	3.8	Neg	—	Neg	Neg
20	23,000	Yes	Yes	—	1.7	Neg	—	Neg	Neg
21	23,000	No	—	3.8	Neg	Neg	Neg	Neg	Neg
22	29,000	Yes	No	48 0	Neg	Neg	Neg	Neg	Neg
23	34,000	Yes	Yes	—	1 6	Neg	—	Neg	Neg
24	40,000	Yes	No	30.1	5.5	Neg	Neg	Neg	Neg
25	40,000	Yes	No	17.0	2.6	Neg	Neg	Neg	Neg
26	50,000	Yes	No	16.1	3.9	Neg	Neg	Neg	Neg
27	50,000	No	—	10.0	2.5	Neg	—	Neg	Neg
28	53,000	Yes	No	2.2	Neg	Neg	Neg	Neg	Neg
29	106,000	Yes	Yes	16.2	1.4	Neg	—	Neg	Neg
30	145,000	Yes	Yes	—	3.1	Neg	—	Neg	Neg
31	7,000	Yes	No	Neg	Neg	Neg	21.8	3.4	Neg
32	19,000	Yes	Yes	Neg	Neg	Neg	2.4	Neg	Neg
33	33,000	No	—	Neg	Neg	Neg	15.4	1.6	Neg
34	39,000	No	—	—	Neg	Neg	—	2 0	Neg
35	44,000	Yes	No	Neg	Neg	Neg	18 2	Neg	Neg
36	69,000	No	—	Neg	Neg	Neg	4.9	1 8	Neg

DISCUSSION

In this report, we describe our experience with two clinically applicable methods for measuring antiplatelet autoantibodies in chronic ITP: the immunobead assay, which is capable of measuring both platelet-associated and plasma autoantibodies, and the microtiter assay, which is useful in detecting only plasma antibodies.

Platelet-associated autoantibodies were more likely to be positive. Positive results were noted in 75% of those tested (21 of 28 patients) and, in six of these, only platelet-associated autoantibodies were demonstrable. Plasma autoantibodies were noted less frequently (57.6% positive, 34 of 59 patients). Autoantibodies to GPIIb/IIIa occurred more commonly than did those to GPIb.

Autoantibody assays have distinct advantages over assays for platelet-associated IgG (PAIgG). First, they allow demonstration of autoantibodies against defined platelet proteins, confirming the autoimmune nature of the patient's disease. Conversely, the PAIgG assay measures IgG on platelets of unknown specificity. Second, autoantibody test results in patients with nonimmune thrombocytopenia have thus far been completely negative with these two assays, whereas PAIgG results are positive in many patients with a variety of diagnoses.[14,15] Although it seems likely, as suggested by the studies of Kelton et al,[15] that positive PAIgG results may reflect immune-mediated platelet destruction, an increase in PAIgG is not diagnostic of autoimmune thrombocytopenia. Although autoantibodies were not seen in the group of thrombocytopenic control patients reported here, when a large number of patients with collagen vascular disease or lymphoma are screened using these assays, positive results may likely be seen in some of these patient groups since they have other types of autoantibodies (eg, anti-RBC antibodies).

The difference in the results between the immunobead assay (36 of 59 positive) and the microtiter-well assay (14 of 59 positive) is of interest. Study of purified protein antigens shows that there are two types of epitopes: sequential and topographic.[13] Sequential epitopes involve amino-acid

Table 4. Antiplatelet Glycoprotein Autoantibodies: Group 3, Positive Microtiter-Well Assay/Negative Immunobead Assay

				Anti-GP IIb/IIIa			Anti-GPIb		
				Immunobead			Immunobead		
Patient	Platelet Count/μL	SPLX	Resp	P-Assoc	Plasma	Well	P-Assoc	Plasma	Well
37	9,000	Yes	No	—	Neg	Neg	—	Neg	Pos(40)
38	10,000	No	—	—	Neg	Pos(320)	—	Neg	Pos(80)
39	56,000	Yes	No	—	Neg	Neg	—	Neg	Pos(160)
40	86,000	Yes	Yes	—	Neg	Pos(640)	—	—	Neg

Table 5. Antiplatelet Glycoprotein Autoantibodies: Group 4, Negative in Both Assays

				Anti-GP IIb/IIIa			Anti-GPIb		
				Immunobead			Immunobead		
Patient	Platelet Count/µL	SPLX	Resp	P-Assoc	Plasma	Well	P-Assoc	Plasma	Well
41	3,000	Yes	No	—	Neg	Neg	—	Neg	Neg
42	6,000	Yes	No	—	Neg	Neg	—	Neg	Neg
43	9,000	Yes	No	—	Neg	Neg	—	Neg	Neg
44	9,000	Yes	Yes	—	Neg	Neg	—	Neg	Neg
45	9,000	Yes	Yes	—	Neg	Neg	—	Neg	Neg
46	18,000	Yes	Yes	—	Neg	Neg	—	Neg	Neg
47	20,000	No	—	—	Neg	Neg	—	Neg	Neg
48	24,000	Yes	Yes	Neg	Neg	Neg	Neg	Neg	Neg
49	34,000	No	—	Neg	Neg	Neg	Neg	Neg	Neg
50	34,000	Yes	No	Neg	Neg	Neg	Neg	Neg	Neg
51	34,000	Yes	No	—	Neg	Neg	—	Neg	Neg
52	40,000	Yes	No	—	Neg	Neg	—	Neg	Neg
53	42,000	No	—	Neg	Neg	Neg	Neg	Neg	Neg
54	42,000	Yes	No	—	Neg	Neg	—	Neg	Neg
55	46,000	No	—	—	Neg	Neg	—	Neg	Neg
56	50,000	Yes	No	—	Neg	Neg	—	Neg	Neg
57	100,000	No	—	—	Neg	Neg	—	Neg	Neg
58	100,000	No	—	Neg	Neg	Neg	Neg	Neg	Neg
59	261,000	Yes	Yes	—	Neg	Neg	—	Neg	Neg

sequences of one section of the protein whereas topographic epitopes involve regions of the molecule remote in sequence but close in three-dimensional space due to the tertiary molecular structure. The most likely explanation for the greater percentage of positivity with the immunobead assay is that solubilization of the platelets prior to incubation with antibody, which is required for the microtiter assay, in some way disturbs the antigenic epitope whereas incubation of antibody and platelets prior to solubilization stabilizes it. We postulate that the microtiter assay measures sequential or stable topographic epitopes while the immunobead assay measures unstable topographic epitopes as well.

Alternatively, the immunoreactivity of the solubilized platelets may be affected by the freezing and thawing of the platelet lysate prior to coating the microtiter wells or binding of the GPs to monoclonal antibody on the microtiter well may alter the molecular configuration. Studies are in progress to evaluate these possibilities.

These assays are both adaptable to the measurement of other as yet unidentified autoantibodies when appropriate monoclonal antibodies become available. Because many patients with chronic ITP have no demonstrable autoantibody, autoantibodies against other platelet-associated antigens (eg, phospholipids, glycolipids, etc.) are probably present.

In summary, the present studies describe the use of two assays for measurement of autoantibodies to specific platelet proteins. Results show that most patients with chronic ITP have autoantibodies against either the platelet GPIIb/IIIa complex or against GPIb. Differences in the frequency of positive results in the two assays provide evidence for epitopes with varying degrees of stability on solubilization.

NOTE ADDED IN PROOF

In recent studies, we have substituted a single 1/4 inch polystyrene bead (Pierce Chemical Co, Rockford, Ill) for the 100 mg of microbeads. Incubations with anti-human IgG and BSA are performed as described, except that we use 10 µg of HB-43 per bead; the beads are washed the same number of times by adding 10 ml of PBS-Tween/bead followed by aspiration of the fluid. After assay completion, the bead is transferred to a clean tube and the radioactivity determined. With this modification, the assay is simpler and the background radioactivity is much lower.

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Survival After Antilymphocyte Globulin Therapy for Aplastic Anemia Depends on Disease Severity

By J.C.W. Marsh, J.M. Hows, K.A. Bryett, S. Al-Hashimi, S.M. Fairhead, and E.C. Gordon-Smith

Sixty-four patients with aplastic anemia were treated with antilymphocyte globulin (ALG Merieux) between 1980 and 1985. The actuarial survival for all patients was 53% at 6 years, with 79% survival for nonsevere aplastic anemia (NSAA) and 36% for severe aplastic anemia (SAA). The neutrophil and platelet counts before treatment with ALG were highly predictive of survival, whereas sex, age, and etiology were not. Duration of disease prior to ALG treatment was inversely related to survival, although this was not statistically significant. Survival was closely associated with response to ALG, especially for patients with SAA. The response to one course of ALG was 33%. Eighteen patients who did not respond to an initial course of ALG received a second course; of these, four (22%) responded. The overall response to one or two courses of ALG was 40%. The mean RBC volume (MCV) measured after ALG treatment was a useful early indicator of response. Both the minimum lymphocyte count during ALG therapy and the mean lymphocyte count after therapy, however, were not significantly different between responders and nonresponders. The disappointing survival of patients with SAA in this study may reflect the poor clinical condition of severely affected patients referred to us and/or the presence of longstanding aplasia. The importance of studying a large series of patients with long-term follow-up to assess ALG in the treatment of aplastic anemia is shown by this investigation.

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TREATMENT OF severe aplastic anemia (SAA) with androgens has not improved the dismal outlook of this disease, with only 20% of patients surviving at 5 years. The prognosis can be improved by allogeneic bone marrow transplantation (BMT) from an HLA-identical sibling donor.[1] BMT is now considered the treatment of choice for SAA, with survivals of 70% to 80% reported.[2-6] For patients who do not have an HLA-identical sibling donor, older patients (>45 years), and for those with nonsevere aplastic anemia (NSAA), however, immunosuppression with antilymphocyte globulin (ALG) has been used increasingly over the last decade. There has been much variation in the reported results of ALG treatment, with survival figures ranging from 35% to 76%.[7-11] Randomized studies confirmed that ALG was more effective than supportive care alone in the treatment of severe aplastic anemia,[11] and that ALG, haploidentical bone marrow, and androgens were more effective than androgens alone.[9] In the UCLA study, 11 of 21 patients who received ALG responded within 3 months, whereas none of 21 control patients improved.[11]

The mechanism of action of ALG in the treatment of aplastic anemia is not known. It may reverse an abnormal immune mediated suppression of bone marrow function[12-14] or induce growth and differentiation of marrow stem cells.[15] The recent work by Zoumbos et al[14] demonstrated the presence in some patients with aplastic anemia of activated suppressor lymphocytes producing interferon, which may have a role in the pathogenesis of bone marrow failure. Further studies directed by these findings may predict which patients will respond to immunosuppressive therapy such as ALG.

We present here an analysis of 64 patients with aplastic anemia treated with ALG at Hammersmith Hospital over a 6-year period, with a median follow-up for survivors of 34 months (range 11 to 70 months). Simple clinical and hematologic parameters have been examined with a view to predicting early response and long-term survival.

MATERIALS AND METHODS

Patients

From 1980 to 1985, 64 patients with aplastic anemia were referred to Hammersmith Hospital and treated with ALG The median age was 25 years (range 3 to 62 years) and the male/female ratio was 1 5 1 In 36% of cases (n = 24) a known etiologic agent was identified. Exposure to drugs was the commonest single cause, of which 3 cases were due to gold therapy, 3 were due to chloramphenicol, and 1 was due to each of the following—phenylbutazone, indomethacin, midazolam, and benoxaprofen. Four cases were due to exposure to chemicals and five were associated with hepatitis. Two patients later developed paroxysmal nocturnal hemoglobuinuria (PNH); three cases were due to each of the following—pregnancy, infectious mononucleosis, and influenza A. Patients with Fanconi's anemia, malignancy, or systemic lupus erythematosis were excluded. Twenty-seven patients had received courses of androgens prior to treatment with ALG. The duration of disease prior to ALG therapy was <2 months in 15 patients, 2 to 10 months in 33 patients, and >10 months in 16 patients. All patients gave informed consent for antilymphocytic globulin therapy or bone marrow transplant procedure in accordance with Hammersmith Hospital ethical guidelines.

Definition of Disease Severity

Patients were classified as having SAA using a modification of the criteria of Camitta et al[16] SAA was defined by two of three of the following peripheral blood criteria being present at the same time: neutrophils $<0.4 \times 10^9$/L, platelets $<20 \times 10^9$/L, reticulocytes $<10 \times 10^9$/L, in association with a hypocellular bone marrow of which <20% of cells were hematopoietic as assessed by bone marrow aspiration. By these criteria, 30 patients (47%) had SAA and the remaining 34 (53%) had NSAA prior to treatment with ALG.

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Immunosuppressive Protocols

All patients received ALG (Institut Merieux). For patients receiving one course only (n = 46), horse ALG was used The remaining 18 patients who did not respond to a first course of horse ALG subsequently received a second course of ALG (rabbit ALG in all but one case). The median time interval between the first and second course of ALG was 6 months (range 3 to 28 months) Horse ALG was prepared in vials containing 50 to 100 mg protein, not less than 4,250 lymphocytotoxicity U/vial,[17] and was given at a dose of 1½ vials/10 kg body weight for 5 consecutive days. The dose of rabbit ALG was 1½ vials/10 kg body weight, each vial containing 25 mg protein. Prednisolone at a dose of 1 mg/kg/day was started on day +6 after ALG treatment to prevent serum sickness; after 1 week, the dose was reduced over the subsequent 14 days. The majority of patients (n = 58) received androgens post-ALG at a dose of 2.5 mg/kg/day for a maximum of ≤6 months, the course being terminated if abnormal liver function developed. If an initial response after ALG was followed by a fall in peripheral blood counts on reducing the dose of oxymethalone, this drug was then continued for >6 months. The remaining six patients received a course of high-dose methyl prednisolone during and continuing after ALG at an initial dose varying from 1.5 to 5 mg/kg/day, gradually reducing to zero over 4 to 8 weeks.

Supportive Therapy

All patients received ALG treatment at Hammersmith Hospital and remained hospitalized for at least 2 weeks after treatment Patients were then treated in the outpatient department unless they required hospitalization for treatment of major infections or control of severe bleeding episodes. Outpatient follow-up was done either at Hammersmith Hospital or jointly at Hammersmith and the referring hospital. Patients were readmitted to Hammersmith Hospital if the referring hospital was unable to manage serious infections or provide adequate platelet support. RBC transfusions were given when clinically indicated or when the hemoglobin concentration dropped to <8 g/dL. Platelets from random donors were given before and after each daily infusion of ALG to maintain the platelet count >20 × 10^9/L. Platelet transfusions were also given if significant bleeding occurred despite higher platelet counts. All patients refractory to platelets from random donors continued to receive HLA-matched platelets. Before ALG treatment was started, patients began taking nonabsorbable oral antibiotics for gut sterilization.[18] Septic episodes (>38°C) were treated with broad-spectrum intravenous (IV) antibiotics, using the current Hammersmith protocols. The same therapy was advised for study patients admitted with infections to peripheral hospitals.

Definition of Response to ALG Treatment

All patients with NSAA and SAA were transfusion dependent prior to therapy with ALG. Response to treatment was defined as complete independence from RBC and platelet transfusions. Response was assessed at 3, 6, and 12 months after treatment with ALG.

Cause of Death

Twenty-six of 64 patients died The commonest cause of death was infection (46% of all deaths), of these, 9 were bacterial, 3 were multiple (bacterial and fungal), but no deaths resulted from viral infection. Acute hemorrhage accounted for five deaths, although several patients who died primarily of infection had evidence of some bleeding at time of death. Six patients died as a result of complications from BMT. One death resulted from each of the following: hepatic carcinoma associated with prolonged use of anabolic steroids, cardiac failure from iron overload, and cavernous sinus thrombosis.

Late Complications

Two patients developed hemolytic PNH, 6 and 26 months, respectively, after ALG following a period of response. One patient has developed a T cell lymphoma 2 years after responding to a second course of ALG. The association of this lymphoma with the aplastic anemia may not be direct.

Statistical Analysis of Results

Actuarial survival curves were calculated using the method of nonparametric estimation for incomplete observations.[19] A log-rank test was used to evaluate the effect of single factors on survival,[20] and the BMDP package[21] was used to perform a Cox regression analysis,[22] which evaluates possible factors prognostic for survival time simultaneously (namely neutrophil count, platelet count, age, sex, etiology, and duration of aplasia). The two-tailed Student's *t* test was used to seek significant differences in simple peripheral blood parameters between responders and nonresponders at different time intervals after the first course of ALG. All data were analyzed up to November 1985.

RESULTS

Survival of Patients

The actuarial survival of all patients with aplastic anemia is 53% at 6 years. Those with SAA had a significantly worse survival of 36% as compared with 79% for NSAA, however, (Fig 1). Eight patients with SAA who showed no response to ALG (5 following one course and 3 following two courses of ALG) were subsequently transplanted (3 from HLA-identical sibling donors and 5 from unrelated HLA-identical donors), of whom 5 are current survivors. In addition, a further four patients who had NSAA at the time of ALG treatment and who did not respond were subsequently transplanted at a time when their disease had progressed to SAA (all from HLA-identical sibling donors). One of these patients is a current survivor. If the transplanted patients are excluded from the analysis, the survival for SAA after treatment with ALG falls from 36% to 26%. Figure 1 shows that the plateau for survival for SAA is not reached until at least 3 years after treatment, although even this may not be a true plateau because the number of patients with SAA surviving after 3 years is small. The median follow-up of survivors for NSAA is 32 months (range 11 to 70 months); for SAA, it is 35 months (range 25 to 69 months)

Factors Predictive of Survival

Univariate analysis of several clinical and hematologic parameters was performed to identify possible predictive factors of survival prior to treatment with ALG. Patients who subsequently received a bone marrow transplant were excluded from the analysis of survival by neutrophil and platelet stratification.

Analysis of survival by neutrophil stratification. Survival by neutrophil stratification (Table 1) showed that patients with a neutrophil count of <0.2 × 10^9/L fared badly

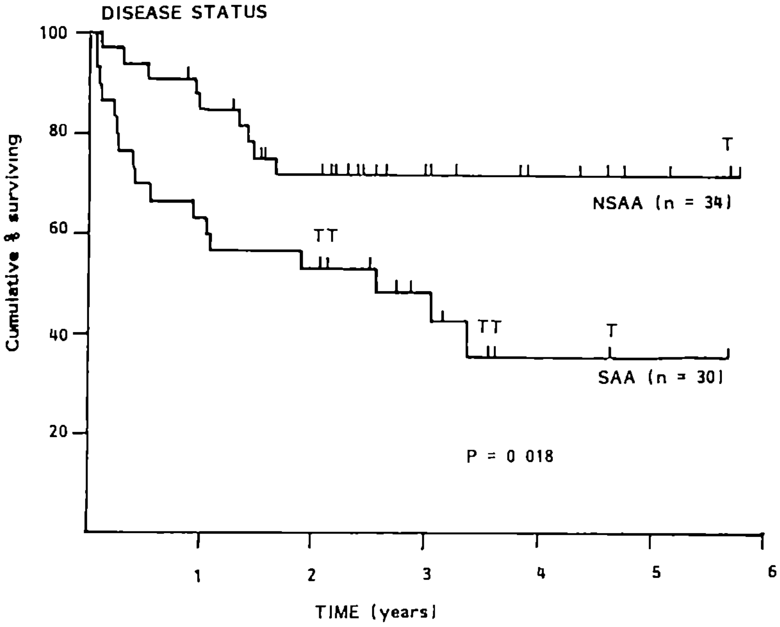

Fig 1. Actuarial survival curve for all patients with aplastic anemia by disease status (SAA, severe aplastic anemia; NSAA, nonsevere aplastic anemia).

(23% survival) as compared with (a) patients having a count of 0.2 to 0.5 × 10^9/L (61% survival, $P = .001$) and (b) patients with neutrophil counts of >0.5 × 10^9/L (75% survival, $P = .0001$).

Analysis of survival by platelet stratification. The platelet count was also a significant variable in predicting survival. Table 1 shows that the survival of patients with an unsupported platelet count >30 × 10^9/L before ALG was significantly better (100% survival) than for patients with a count <10 × 10^9/L (38% survival, $P = .013$).

Table 1. Analysis of Pretreatment Patient Characteristics and Survival

	No of Patients	Survival (%)
Factors shown to predict survival		
Neutrophils × 10^9/L*		
<0 2	13	23
0 2–0.5	14	61
>0.5	25	75
Platelets × 10^9/L		
<10	15	38
10–30	27	53
>30	7	100
(Patients who subsequently received a bone marrow transplant have been excluded from the above data.)		
Factors shown not to predict survival		
Sex		
M	38	44
F	26	58
Age (yrs)		
<15	18	50
15–25	14	64
>25	31	49
Etiology		
Idiopathic	41	46
Drugs	10	60
Hepatitis	5	40
Disease duration (mos)		
<2	15	76
2–10	33	49
>10	16	43

*Details of statistical significance are described in text

Factors not predictive of survival. Factors shown not to influence survival significantly were sex, etiology, and age (Table 1). The duration of disease before treatment with ALG was inversely related to survival (Table 1). The survival of patients who had their disease for <2 months was 76% as compared with 43% if disease was of >10-month duration, although this difference was not statistically significant ($P = .095$).

Multivariate analysis of the following factors (neutrophil count, platelet count, age, sex, etiology, and duration of aplasia) showed that the pretreatment platelet count ($P = .0012$) and neutrophil count ($P = .027$) were independently significant factors in predicting survival after ALG therapy.

Analysis of Response to ALG Treatment

The outcome of patients treated with one or two courses of ALG is shown in Figs 2A and B and 3. After one course of ALG, nine patients had responded by 3 months, eight by 6 months and four were late "responders" (response by >6 months), giving an overall response rate of 21 of 64 patients (33%). Six nonresponders were subsequently transplanted (two other patients received a BMT <3 months after ALG), and 16 patients died after receiving one course of ALG. Of those who did not respond to one course of ALG, 18 of 44 received a second course (Fig 3). These included two patients who initially responded to a first course, but later relapsed. After a second course, 4 of 18 patients had responded by 12 months (response rate 22%). A response to one or more courses of ALG occurred in 25 of 64 patients (40%). The majority of responders to the initial course of ALG (n = 18 of 21, 88%) had NSAA, as did all four patients responding

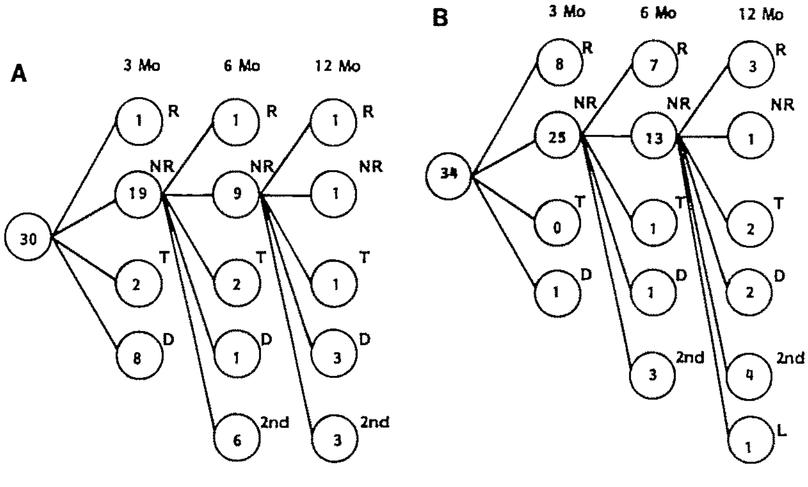

Fig 2. Outcome of patients (A) with severe aplastic anemia and (B) with nonsevere aplastic anemia treated with one course of ALG. R, responders; NR, nonresponders; T, transplanted; D, deaths; 2nd, second course of ALG.

after two courses. Just prior to receiving their second course, however, two of the responders whose disease was initially classified as NSAA had progressed to SAA. Three patients were transplanted at 6 months after their second course of ALG (2 with SAA and 1 who initially had NSAA but whose disease had progressed to SAA), and a further patient with SAA was transplanted following no response to a third course of ALG. There were 4 more deaths (2 with SAA and 2 with NSAA), 3 of these having shown no response by 6 and >12 months (Fig 3) and 1 with SAA who died at 2 months after the second course of ALG. There was good correlation of survival and response, with 100% of responders surviving as compared with 37% survival for nonresponders.

Analysis of Possible Early Indicators of Response After Treatment

In an attempt to predict at an early stage which patients would respond after receiving a course of ALG, several hematologic parameters were examined and compared in responders and nonresponders. Patients who subsequently received a BMT were excluded from this analysis

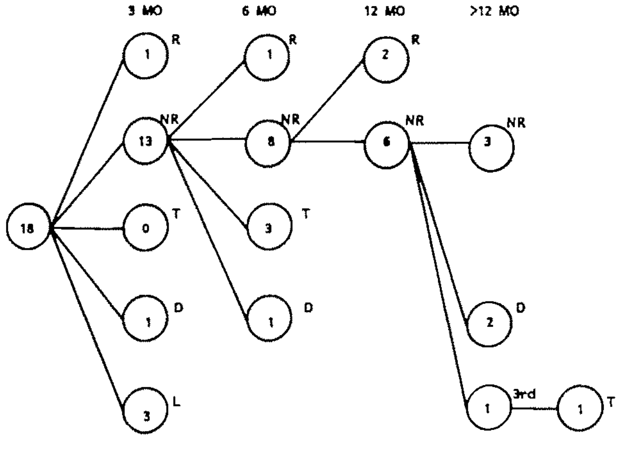

Fig 3. Outcome of all patients (severe and nonsevere aplastic anemia) treated with two courses of ALG. R, responders; NR, nonresponders; T, transplanted; D, deaths; 3rd, third course of ALG; L, lost to follow-up. The total number of patients receiving a second course of ALG includes two patients who responded to a first course but later relapsed.

Lymphocyte count. Although the mechanism of action in the treatment of aplastic anemia is unknown, ALG administration does result in a reduction in peripheral blood lymphocytes. It was therefore of interest to see if the mean lymphocyte counts during and after treatment could predict response to ALG. Figure 4A shows that there was no significant difference in mean lymphocyte counts at various time intervals after treatment in responders and nonresponders. Indeed, unexpectedly, responders tended to have somewhat higher counts than nonresponders. In addition, there was no significant difference in mean lymphocyte counts between the two groups on each of the 5 days of treatment.

Mean RBC volume. The mean RBC volume (MCV) was significantly higher in responders at 1 week after ALG treatment (Fig 4B) with increased significance at 1 month, thus providing a useful early predictor of response to ALG. The mean neutrophil and platelet counts after ALG therapy were also significantly higher in responders, but the great degree of overlap of SD precluded its use as predictors of response.

DISCUSSION

In 1970, Mathé first reported the use of ALG as immunosuppressive therapy followed by infusion of haploidentical bone marrow in the treatment of aplastic anemia.[23] Speck et al in 1977 reported a 2-year survival of 70% after ALG for patients with SAA, and the same workers later showed that it was not necessary to give concurrent haploidentical marrow.[7] Other studies, however, have produced less impressive results. An earlier study from Hammersmith[8] (in which 5 of the 30 patients studied are also included in this present series) reported an overall survival of 52% at 2 years for all patients with aplastic anemia after ALG treatment but only 35% for those with SAA. A high proportion of these patients had a prolonged history of aplasia. An analysis by the European Bone Marrow Transplant Severe Aplastic Anaemia Working Party (Bacigalupo et al[10]) in the treatment of >300 patients with SAA in Europe from 1970 to 1983 revealed 48% survival at 9 years after treatment with

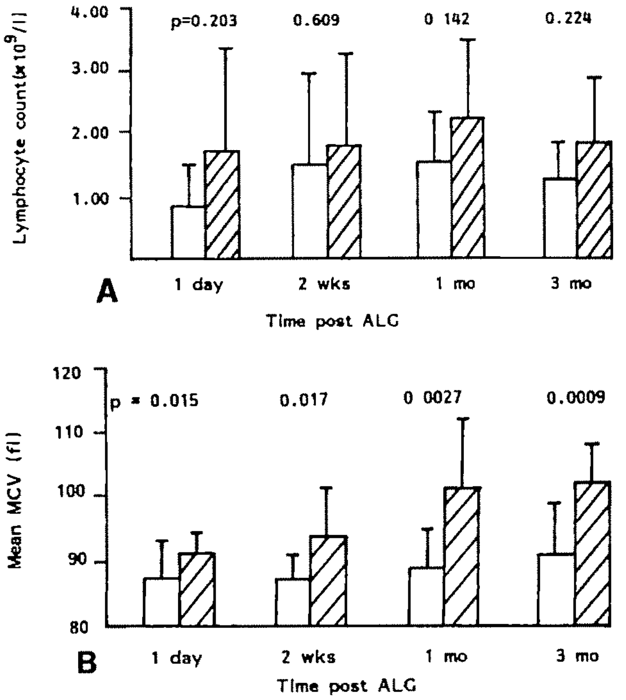

Fig 4. (A) Analysis of lymphocyte count in responders (n = 9) and nonresponders (n = 43) at 3 months. (B) Analysis of mean RBC volume (MCV) in responders (n = 9) and nonresponders (n = 43) at 3 months.

ALG. Furthermore, survival was not influenced by the year of treatment, there being no improvement in survival figures during the latter 10 years of study. It was not until 1983 that a prospective randomized study was performed[11] demonstrating 52% partial or complete remission at 3 months after ALG therapy for patients with moderate or severe aplastic anemia as compared with no remission in matched patients receiving blood products and supportive care alone.

In our study of 64 patients, the overall response rate was 40%. The survival of NSAA (79% at 6 years) contrasted significantly with those with SAA (36%). Furthermore, the actuarial survival of patients with SAA fell to 26% when eight patients who were subsequently treated by BMT were excluded from the study. Therefore, any assessment of ALG in the treatment of aplastic anemia should clearly differentiate between severe and nonsevere disease. Nevertheless, with cases of NSAA excluded, there is great variation in results for treatment of SAA with ALG from various centers. Our results for SAA appear to be worse than in previously reported studies,[7-11] perhaps for the following reasons. Patients referred to the Hammersmith Hospital were often heavily sensitized to blood products and therefore refractory to random platelet transfusions or were seriously ill from severe infection. As a result, a large percentage of patients required intensive supportive care, necessitating a delay before ALG treatment was started. Although not statistically significant, there was a trend for patients who had their disease for >10 months prior to ALG treatment to fare worse than those with disease for <2 months (Table 1). A worse prognosis for patients with long-established disease was also suggested by the UCLA trial,[11] although disease duration was shown to have no effect by Doney.[24] In contrast, the European study (1982) reported that a longer diagnosis–treatment interval was associated with better survival as compared with those with shorter duration of disease. They were all patients with less severe disease, however.[25] Many patients referred to the Hammersmith Hospital were very severely neutropenic, and of the patients with SAA in this study, 50% had neutrophil counts of $<0.2 \times 10^9/L$, which is associated with poor survival after ALG (Table 1). More recent data from a larger analysis of patients with SAA treated with ALG throughout the United Kingdom has confirmed the poor outlook of those with very severe aplastic anemia as defined by neutrophil count $<0.2 \times 10^9/L$.[26] In addition, prior to treatment with ALG, 42% had platelet counts $<10 \times 10^9/L$, another factor that we find predicts poor survival (Table 1). It may be necessary to introduce new criteria to define the very severe subgroups of SAA identified in our study.

The criteria used for defining response to ALG vary between centers. We have used strict criteria in that only those patients who become completely independent of RBC and platelet transfusion have been classified as responders. This is a simple, practical definition of response and is justified by the good correlation between response to ALG treatment and survival in this study (described in section on Analysis of Response to ALG Treatment). Some patients classified as responders in other studies[7-9,11] would have been excluded from our definition of a response to ALG became of continued transfusion support.

The importance of studying a large number of patients with long-term follow-up cannot be overemphasized. To quote a 50% actuarial survival figure at 2 years for SAA from our results would have been misleading (Fig 1), and a median of 34 months follow-up appears to be the minimum time for reporting survival of patients with SAA treated with ALG.

Variability in results between different centers using ALG may be due to other factors. There has been inconsistency in the use of androgens in patients treated with ALG. Gluckman et al[25] showed that androgens did not influence the response rate to ALG and, in a prospective randomized trial, Champlin[27] demonstrated that androgens do not alter response after ALG therapy. Recent data from a multicenter European study,[28] however, showed a significantly better survival among patients with SAA who received ALG with both androgens and high-dose methyl prednisolone as compared with those receiving the same schedule without androgens. In our study, there was no significant difference in survival for patients with SAA treated with ALG with or without androgens (data not shown).

Androgens are of benefit to some patients with NSAA because 25% to 50% of patients relapse after discontinuation of the drug, indicating androgen dependence,[29,30] and we accept that some of the patients with NSAA in our study may have responded to the androgens rather than to ALG. The use of androgens in combination with ALG for treatment of aplastic anemia is controversial, and further randomized studies are needed to address this important point. Likewise, the additional use of cortisteroids at low or high dose with ALG has no effect on survival.[10]

A lack of standardization of ALG therapy itself may contribute to differences in response rates. In addition to different doses and duration of therapy, there appears to be variability in the efficacy of ALG used from different sources. There are differences in protein content of various preparations of ALG, but more important are differences in specific activity as measured by lymphocytoxicity testing in vitro. Furthermore, Smith et al,[31] have characterized the antibody specifications of two batches of Swiss ATG using a panel of monoclonal antibodies that recognizes T cell antigens. They showed that the different batches had differing antibody specificities which might have contributed to the difference in response rate observed in two separate trials each using one of these two batches of Swiss ATG for treatment of SAA. More recently, however, Gratwohl[31a] showed in a retrospective analysis that although variations existed in the preparation and immunologic properties of ALG, batches prepared at different times over a 10-year period were not associated with any corresponding difference in outcome of patients with SAA treated with ALG.

Measurement of MCV is a useful, simple, and early predictor of response to ALG treatment. The observation that responders had a significantly higher mean MCV is merely a reflection of endogenous production of RBCs and not of any qualitative difference between RBCs produced in responders and nonresponders.

The value of giving two courses of ALG to a patient with aplastic anemia has not previously been assessed. We have shown that 22% of patients not responding to an initial course will achieve a response to a second course of ALG within 12 months, although it is impossible to determine whether this "response" might instead be a late "response" to the first course of ALG or even spontaneous recovery. Having established from our study that the MCV is an early predictor for response, we may be able to select the patients who would benefit from an early second course of ALG 2 to 3 months after the first course. No major allergic side effects occurred with the second course. The lack of toxicity from a second course of ALG may be due to the use of a different animal source. The incidence of relapse after response to ALG was low, occurring in only two patients. Both patients responded to a second course, and one has subsequently responded to two additional courses given for recurrent relapse. Thus, improvements in response might be achieved by giving multiple courses of ALG.

We have shown that a convincing plateau of survival may not be achieved by 3 years after ALG therapy for SAA [especially if the patient has $<0.2 \times 10^9/L$ neutrophils or $<10 \times 10^9/L$ platelets (Table 1, individual survival curves not shown)]. We have confirmed this in a larger, more recent study of ALG treatment for aplastic anemia in the United Kingdom.[26] These findings are also in agreement with recent data from the European Bone Marrow Transplant Severe Aplastic Anemia Working Party, 1986.[28] Alternative forms of treatment should be sought for those patients with severe aplastic anemia without an HLA-identical sibling marrow donor There has been only limited experience in the use of HLA phenotypically matched unrelated donors,[32,33] or HLA-mismatched family donors[34] for BMT in SAA. A pilot study from the Hammersmith Hospital of eight unrelated histocompatible and five related HLA-mismatched transplants for SAA indicates an overall actuarial survival of 39% with follow-up from 3.2 to 4.7 years, however (median 4.2 years).[35] Nine of 13 patients in this group had previously received ALG without response. Although BMTs from unrelated or mismatched family donors are less successful than HLA-identical sibling transplants, this approach may offer a reasonable chance of cure for younger patients who have failed to respond to immunosuppressive therapy.

We recommend that patients diagnosed as having aplastic anemia should be referred as soon as possible for treatment, the latter depending on disease severity, age, and potential availability of an HLA-identical sibling donor.

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A Monoclonal Anti-Human Plasma Prekallikrein Antibody That Inhibits Activation of Prekallikrein by Factor XIIa on a Surface

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Of five IgGl/k murine monoclonal anti-human prekallikrein antibodies produced (MAbs), MAb 13G11 was selected for studying interaction of prekallikrein with factor XII and high-mol-wt kininogen (HMWK) during activation on a surface. Immunoblots from sodium dodecyl sulfate (SDS) gels showed that this MAb recognizes two variants (88 kd and 85 kd) of prekallikrein and kallikrein both in purified proteins and normal plasma. Under reducing conditions, kallikrein exhibits the epitope on the heavy chain but not on the light chains. Preincubation of MAb 13G11 with prekallikrein (added to prekallikrein-deficient plasma) or with normal plasma inhibited surface activation of prekallikrein 60% to 80%, as judged by amidolytic and coagulant assays. In normal plasma, inhibition by the Fab fragments was 87% of that with the entire MAb. Inhibition was not by competition between the MAb and HMWK, since neither binding of 13G11 to prekallikrein (coated on microtiter plates) was inhibited by an excess of HMWK, nor was hydrolysis of HMWK by kallikrein inhibited by 13G11. Using purified proteins in a system mimicking contact activation, inhibition by 13G11 of prekallikrein activation by factor XIIa, HMWK, and kaolin present was ~80%. Decreased inhibition (55% to 25%) occurred without HMWK or when kallikrein was used instead of prekallikrein. Kallikrein activity was not inhibited by 13G11 Fab fragments. These results indicate that the effect of 13G11 in plasma was neither dissociation of prekallikrein–HMWK complex nor a direct effect on kallikrein activity. Similar to the results in plasma, activation of prekallikrein, HMWK present, by factor XIIa bound to kaolin, was inhibited ~70% by 13G11. The results suggest a previously unrecognized site on the prekallikrein (heavy chain) required for its interaction with factor XIIa, either shared with the 13G11 epitope or located in very close proximity. The inhibition of kallikrein by intact 13G11 indicates that its binding site on the heavy chain is sterically related to the active site (light chain).

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HUMAN prekallikrein, a serine protease zymogen, is present in plasma in two variants (88 kd and 85 kd), probably due to different carbohydrate content.[1] Because ~75% of the total zymogen circulates in plasma noncovalently associated with high-mol-wt kininogen (HMWK),[2,3] the prekallikrein–HMWK complex and not free prekallikrein is probably the biologically relevant species in most of the reactions. Indeed, during the contact activation reactions, HMWK mediates the binding of prekallikrein to a negatively charged surface[4] required for its optimal activation to kallikrein[5,6] by activated factor XII (factor XIIa). Factor XII, which is slowly autoactivated on its binding to the same surface[7,8] is more rapidly cleaved by kallikrein in a positive feedback reaction to produce more factor XIIa and XIIf.[9] Kallikrein consists of a light chain (35 kd or 38 kd), which exhibits proteolytic activity similar to that of pancreatic trypsin and probably originated several billion years ago[10] and a heavy chain (50 kd) of more recent evolutionary origin.[11,12] The light chain is bound by a disulfide bridge(s) to the heavy chain which is essential for specific pathophysiologic functions such as in vitro coagulation,[13] fibrinolysis,[14,15] neutrophil activation,[16] and release of bradykinin.[17] Although kallikrein light and heavy chains can behave as functionally independent domains,[13] the interaction between these two chains on binding of the protease to a surface,[18] during coagulation and cleavage of HMWK,[13] and neutrophil aggregation and degranulation,[19] is essential for full biologic activity. For example, a single cleavage in the heavy chain can affect the catalytic activity inherent to the light chain.[19] Binding of HMWK to the NH_2-terminal region of prekallikrein (kallikrein heavy chain)[13] can lead to alteration throughout the molecule since determinations of functional and immunologic prekallikrein are dependent on the presence of that contact cofactor.[3] During contact activation, interaction between prekallikrein and/or kallikrein with HMWK on a negatively charged surface with resultant acceleration of the process has been reported.[4,6,20] This acceleration has been hypothesized to be caused by placement of prekallikrein–HMWK in an optimal position for its interaction with factor XIIa[20] or in a favorable conformation for activation by activated factor XII.[4]

For a better understanding of the interactions between prekallikrein and the other proteins of the contact activation, monoclonal antibodies (MAbs) to plasma prekallikrein (6A6, 13H11, 13G11, 10B6, and 7H2) have been produced; 6A6, 13H11, and 13G11 have been characterized. MAb 13G11 was selected as a probe in these studies both in plasma and in a system containing purified proteins, which mimics the contact activation system. This MAb appears to react with an epitope on the heavy chain involved in the interaction between prekallikrein and factor XIIa during the activation of prekallikrein on a surface. This is the first report on development and use of monoclonal antibodies against human plasma prekallikrein.

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MATERIALS AND METHODS

Plasma proteins. For prekallikrein purification, fresh blood (4 U) was collected in tubes containing one tenth of its volume of a solution consisting, per 100 mL, of 3.8 g sodium citrate, 2.5 g dextrose, 0.37 g EDTA, 0.16 g benzamidine, 3 TIU aprotinin (Sigma Chemical, St Louis), 10 mg hexadimethrine bromide, and 40 mg soybean trypsin inhibitor. The plasma obtained was treated with diisopropyl fluorophosphate (final concentration 0.2 mmol/L) and 3 TIU of aprotinin. Purification was accomplished as previously reported[21] with some modifications. In brief, after adsorption of acidic proteins, such as HMWK, to QAE-Sephadex A-50, prekallikrein purification was by two consecutive SP-Sephadex C-50 chromatographies followed by affinity chromatography for removal of IgG. Equilibration of the QAE-Sephadex was with the Tris buffer[21] containing 50 μg/mL hexadimethrine bromide except for the third QAE-Sephadex treatment. Before this last treatment, 1.5 mol/L urea (final concentration) was dissolved in the filtrate of the previous QAE-Sephadex to facilitate dissociation of plasma proteins such as HMWK. Washing after each QAE treatment with the buffer was monitored for prekallikrein. The final filtrate, after dilution to the desired conductivity, was stirred for 75 minutes at 4°C with 700 mL SP-Sephadex slurry made with the above Tris buffer, which was then loaded into a column. The fractions corresponding to the eluted peak containing prekallikrein were pooled, concentrated, adjusted to the ionic strength of the equilibration buffer, and applied to the second SP-Sephadex column, followed by protein elution as previously reported.[21] The fractions of the prekallikrein peak were pooled either as described[21] or in eight portions each corresponding to one eighth of this peak, to be used, after removal of IgG, for mouse immunization and hybridoma supernatant screening or structural and functional assays, respectively. In agreement with a higher pI for the 85-kd prekallikrein,[1] the first eluted fractions contained mainly the 88-kd prekallikrein with increasing amounts of the 85-kd variant as elution proceeded. These fractions were adjusted to pH 5.8 and stored at −70°C. An IgG precipitate formed during storage was removed by centrifugation. The supernatant was concentrated about fivefold, adjusted to pH 7.5 with 1 mol/L Tris and applied to two columns placed in tandem, protein A-Sepharose (bed volume 3 mL) and anti-(human-IgG)-agarose (bed volume 6 mL). Elution was with 10 mmol/L Tris-HCl/0.15 mol/L NaCl pH 7.4 (TBS). The fractions containing prekallikrein were pooled, concentrated to 0.1 to 0.5 mg protein/mL and stored at −70°C. Only the first eluted fractions containing mainly the 88-kd variant and not exhibiting contaminations with factor XI[21] were used in the experiments. The concentrated prekallikrein exhibited amidolytic activity, as determined with D-Pro-Phe-Arg-pnitroanilide (D-Pro-Phe-Arg-pNa), which was 4% of that present when the same prekallikrein was fully activated with factor XII fragment (described below). The kallikrein thus formed hydrolyzed 85 μmoles D-Pro-Phe-Arg-pNa/min/mg determined as described below.

HMWK was purified from the proteins adsorbed to the QAE-Sephadex (described above) as previously described.[22] Factor XII was purified by the method of Fujikawa and Davie.[23] Concentrations of purified HMWK and factor XII were determined by the coagulant activities as reported, HMWK[3] and factor XII,[19] using normal pooled plasma (20 individuals) as the standard and assuming 80 μg HMWK and factor XII 30 μg/mL of normal plasma. The specific activities of these proteins, expressed as coagulant units per milligram, were 12 and 33 for HMWK and factor XII, respectively. The purity of factor XII and HMWK was assessed by sodium dodecyl sulfate (SDS)-gels electrophoresis (10% acrylamide) using Laemmli method,[24] under nonreducing and reducing conditions. Under these conditions, one band comprising ~95% of total protein was observed for factor XII (80 kd) and HMWK (120 kd). Because HMWK contained a trace contaminant that cleaved prekallikrein in the presence of kaolin and was inhibited by corn trypsin inhibitor, this inhibitor was used, as described, in some experiments.

Production of MAbs. MAbs were produced at the Hybridoma Center, Temple University, Philadelphia. In brief, four BALB/c AnSkh mice were immunized subcutaneously (SC) with 35 μg prekallikrein/mouse in complete Freund's adjuvant (week 0) and then again subcutaneously with 35 μg of protein/mouse in incomplete Freund's adjuvant at week 5. At week 11, 50 μg immunogen in 0.15 mol/L NaCl/mouse were injected intraperitoneally (IP). Four days later, blood was screened for antibody production. The two strongest positive mice were selected as spleen donors for fusion. Splenocyte fusion with SP2/O-AG 14 myeloma cells, selection, and growth of hybridomas was basically as reported by McKearn.[25] Concanavalin A-coated tissue culture dishes used for cell fusion and concentration of splenocytes and myeloma cells added to each fusion dish were as described.[26] Cell culture supernatants were screened with a standard enzyme-linked immunosorbent assay (ELISA) using purified prekallikrein as the antigen. Hybridoma cultures with antibody reactive to the immunogen were subcloned twice by limiting dilution and were retested by ELISA for antibody production. Cells from the five strongest positive cultures were injected IP (~2×10^6 cells in 0.5 mL phosphate-buffered saline (PBS)/mouse) into five BALB/c mice primed 10 to 14 days previously with 0.5 mL pristane for antibody production.

Antibody purification and Fab fragment preparation. For MAb purification, the ascites fluid (0.5 to 1.0 mL) was desalted by gel filtration (PD-10 columns, Pharmacia, Piscataway, NJ), according to manufacter's instructions, using a 20 mmol/L Tris-HCl pH 7.8 buffer. The protein fraction was applied to a Mono-Q prepacked HR 5/5 ion exchange column (Pharmacia) adapted to a fast protein liquid chromatography system (FPLC, Pharmacia) consisting of two model P-500 pumps, a Single Path Monitor UV-1 (control and optical units). a single-channel recorder Rec-481, a fraction collector Frac-100, a valve V-7, and a model GP-250 Gradient programmer. The protein was eluted (1 mL/min) by a step gradient generated by buffer A (20 mmol/L Tris-HCl, pH 7.8) and buffer B (20 mmol/L Tris-HCl/1.0 mol/L NaCl, pH 7.8). Programming was planned to give an isocratic elution (90 mmol/L NaCl) of the monoclonal antibody. The antibody fractions were detected with mouse anti-IgG by double immunodiffusion. The immunochemically positive fractions were rechromatographed by the same technique, except that buffers were pH 7.5. The MAbs were then concentrated (PM-10) to 1 to 2 mg/mL and stored at −70°C. MAbs thus purified were used in all assays.

For Fab preparation, MAb 13G11 (6 mg) in 7 mL of 10 mmol/L sodium phosphate/150 mmol/L of NaCl, pH 7.4, containing 1 mmol/L EDTA, and 8.6 mmol/L mercaptoethanol, were incubated with 69 μg of papain (Sigma) for 60 minutes at 37°C under N_2 in the dark. After completion of the hydrolysis as determined by gel filtration, the reaction was stopped by addition of iodoacetamide to a final concentration of 20 mmol/L, followed by incubation for an additional 15 minutes. The Fab and Fc fragments were then separated either with a Superose 12 prepacked HR 10/30 gel filtration column (Pharmacia) using as the buffer 20 mmol/L Tris-HCl/90 mmol/L NaCl pH 7.5 or with the Mono Q column using the procedure above.

Chromatofocusing. Chromatofocusing was carried out with the FPLC system using a Mono P HR 5/20 column (Pharmacia), 100 to 200 μg MAbs, 0.025 mol/L imidazole-HCl pH 7.4 as the starting buffer and diluted 1:10 Polybuffer 74-HCl pH 4.0 as eluent buffer, according to the manufacturer's instructions. The pH gradient was from pH 7 to pH 4.

Protein determination. Protein concentrations were determined according to the method of Lowry et al.[27] using as standard human

IgG for the MAbs and bovine serum albumin (BSA) for the other plasma proteins.

Electrophoresis. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out as described,[24] using 10% acrylamide. Protein standards (BioRad), a mixture of myosin (200 kd), β-galactosidase (116 kd), phosphorylase B (93 kd), BSA (66 kd), ovalbumin (45 kd), carbonic anhydrase (31 kd), soy bean trypsin inhibitor (22 kd), and lysozyme (14 kd) were used.

Western blotting. Electrophoretic transfer onto the nitrocellulose sheets (0.45-μm pore size, Schleicher and Schuell) of the proteins separated by SDS-PAGE was at 8V/cm (with respect to electrode separation) for 16 to 20 hours, at 4°C, according to the reported method.[28] In some experiments, protein solutions (0.2 to 0.5 μg, 0.2 to 0.5 μL) were directly applied to the nitrocellulose membrane and air dried. The nitrocellulose sheets, after brief washing with TBS, were incubated with TBS containing 1 mg/mL BSA for 3 hours at room temperature followed by incubation with the MAbs (0.2 to 10 μg/mL TBS-BSA) for 3 hours. The protein blot was washed with four changes of TBS-BSA containing 0.05% Nonidet P-40 and incubated with goat anti-mouse IgG-conjugated with horseradish peroxidase (0.040 purpurogallin U/mL, Sigma). The protein blots were washed as previously with an additional washing with TBS and then placed in a solution of TBS/methanol (100 mL/20 mL) containing 0.015% H_2O_2 and 0.05% 4-chloro-1-naphtol for 15 to 30 minutes, for detection of the peroxidase-conjugated IgG. When the starting material was plasma, electrophoresis and Western blotting were carried out in the same way except that 5 μL plasma were used and casein (5%), adjusted to pH 7.5, was used instead of BSA.

Antibody subtyping. This determination was carried out with both a Mouse Immunoglobulin Subtype Identification Kit (Boehringer Mannheim Biochemicals) and a MonoAb-ID EIA Kit (Zymed Laboratories, Inc.) after the microtiter plates were coated with prekallikrein (0.2 μg/200 μL of 0.05 mol/L sodium carbonate-bicarbonate pH 9.6 per plate well), at 4°C for 16 to 20 hours.

ELISA assays. Binding of prekallikrein 0.2 to 0.5 μg/microtiter plate well (EIA cuvettes, Gilford, Oberlin, Ohio) and detection of the antibody bound to the immunogen was as described,[29] except that Nonidet P-40 was used instead of Tween 20.

Procoagulant activity of prekallikrein. Prekallikrein activation and coagulant activity of the kallikrein formed was as described[19] using the prekallikrein-deficient plasma graciously supplied by Dr Charles Abildgaard, Davis, CA.

Determination of concentrations of purified prekallikrein and kallikrein. Concentrations of these proteins were determined before each experiment. When prekallikrein was the starting material, an aliquot of this zymogen was diluted in TBS containing human serum albumin (HSA) 1 mg/mL to make a solution of prekallikrein 10 to 20 μg/mL, was activated by incubation with factor XII fragment (1 enzyme/100 prekallikrein, wt/wt) for 30 minutes at 37°C. At this time, maximum activation was reached. Factor XII fragment was then inactivated with corn trypsin inhibitor (10 inhibitor/factor XII fragment, wt/wt). Kallikrein thus obtained was that used in the experiments. Kallikrein activity was determined in D-Pro-Phe-Arg-pNa (1 mmol/L) made in 0.1 mol/L sodium phosphate/150 mmol/L NaCl, pH 7.6. Release of *p*-nitroaniline was followed spectrophotometrically at 405 nm. Calculations were based on the reported molar extinction coefficient,[30] 10 mmol L^{-1} cm^{-1}. Enzyme activity of active protein was normalized to an arbitrary value of 85 μmol of D-Pro-Phe-Arg-pNa hydrolyzed/min/mg protein at 37°C. Based on this value and on that of kallikrein activity determined as described,[31] prekallikrein concentration in plasma was 25 μg/mL.

Incubation of MAbs with plasma followed by activation with kaolin. Incubation of MAbs with purified prekallikrein (to be added to prekallikrein-deficient plasma) or normal plasma was for 40 minutes at 37°C. After incubation, the MAb-normal plasma mixture or MAb-prekallikrein in the prekallikrein-deficient plasma was mixed for 5 seconds (vortex) with a kaolin suspension followed by incubation for 55 seconds at room temperature and amidolytic determination as described above. Kaolin suspensions (1 mg/mL) were made in 0.1 mol/L sodium phosphate/0.15 mol/L NaCl, pH 7.6 or 0.1 mol/L Tris-HCl/0.15 mol/L NaCl, pH 8.0 depending on the substrate used for amidolytic activity, D-Pro-Phe-Arg-pNa or Bz-Pro-Phe-Arg-pNa.

SDS-PAGE analysis of the effect of MAb 13G11 on HMWK hydrolysis by kallikrein. Two polypropylene microtubes, each containing kallikrein (0.6 μg, 9 μL) and 10 μL (10 μg) monoclonal antibody to factor XI or MAb 13G11 were incubated at 37°C for 40 minutes. HMWK (128 μg, 140 μL) and corn trypsin inhibitor (10 μg, 4 μL) were then added to each incubation mixture. To an aliquot (10 μL), withdrawn from each incubation mixture, 35 μL of TBS, 4 μL of mercaptoethanol, and 15 μL of sample buffer[27] were added before analysis by SDS-gels (zero time incubation). The two remaining incubation mixtures were further incubated for 5, 10, 15, 20, 30, and 45 minutes and then treated as described for the zero time incubation.

Purified system mimicking contact activation. The effect of MAbs on prekallikrein activation or kallikrein activity were tested using a system that mimics contact activation. To test the effect on prekallikrein activation each assay mixture contained prekallikrein (0.8 μg, 12 μL), HMWK (2 μg, 2 μL), kaolin suspension (20 μg, 20 μL) made in 0.1 mol/L Tris-HCl/0.15 mol/L NaCl, pH 7.5, and various quantities of MAb in a final volume adjusted to 52 μL. The reaction started by addition of factor XII (0.24 μg, 2 μL) followed by incubation at 37°C for 30 minutes, which was predetermined to be the time for maximal activation in the absence of MAb. Determination of protease activity with D-Pro-Phe-Arg-pNa was then carried out as described above. Assays were also carried out under the same conditions except that HMWK was absent. To test the effect of

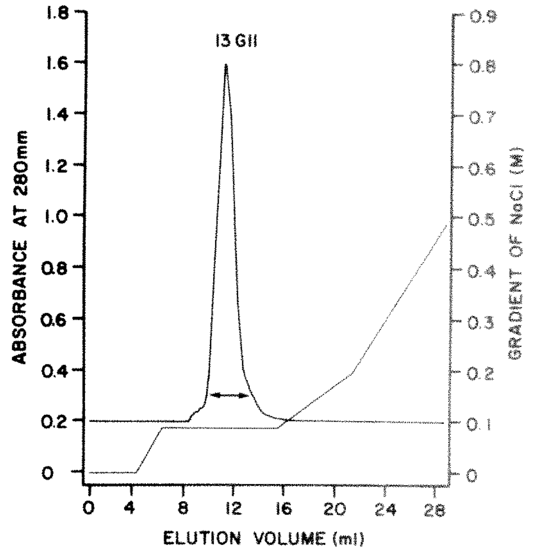

Fig 1. Ion-exchange rechromatography of MAb 13G11 by FPLC. MAb 13G11 was purified from ascites fluid (0.5 mL) by ion-exchange chromatography (Mono-Q column) in a FPLC apparatus. Rechromatography of the fractions containing the antibody using a step gradient (0% to 100% of buffer B) was monitored by absorbance at 280 nm. The fractions collected containing MAb 13G11 are indicated. Details are given in the Materials and Methods section.

MAb 13G11 on kallikrein activity (activated system), prekallikrein was activated before MAb addition in a system as described above except that MAb was absent. After incubation, MAb was added followed by further incubation for 40 minutes and determination of residual activity. Results, expressed as percentage of inhibition, were calculated on the basis of the activity, in the absence of antibody (control), as 100% (or 0% inhibition).

Preparation of a suspension of factor XIIa bound to kaolin. Autoactivation of factor XII was as follows. Factor XII (2.4 μg) in 20 μL of 35 mmol/L Tris-HCl/50 mmol/L NaCl pH 8.0 containing 0.2 mg HSA was mixed for 5 seconds with 50 μL kaolin suspension (2 mg/mL 50 mmol/L Tris-HCl/75 mmol/L NaCl, pH 8.0) and incubated at 37°C for 90 minutes (maximal factor XII autoactivation). The mixture was then centrifuged for 5 minutes using a Fisher microcentrifuge Model 235A. The pellet was washed three times with 20 mmol/L Tris-HCl/90 mmol/L NaCl, pH 7.5. Before the third washing, 25 μg of a suspension of 1 mg kaolin/mL of the buffer were added. The washed pellet was suspended in 150 μL of buffer containing 0.2 mg HSA. Incubation time for maximal activation of factor XII was determined in the following way: 20-μL aliquots of the mixture incubated from 0 to 150 minutes were mixed with 0.3 mL 0.5 mmol/L D-Pro-Phe-Arg-pNa containing polybrene (5 μg/mL) and soybean trypsin inhibitor (50 μg/mL) followed by further incubation for 180 minutes. After the reaction was stopped by addition of 150 μL of 20% of acetic acid, *p*-nitrophenol release was measured spectrophotometrically.

RESULTS

Purification, subtyping, and selection of MAbs. Among the MAbs produced, 6A6, 13H11, 13G11, 10B6, and 7H2 exhibited strong reactivity to prekallikrein as analyzed by ELISA. Purification by ion-exchange rechromatography showed that MAbs 13H11 and 13G11 eluted as a single peak (shown for 13G11, Fig 1) and 6A6, 10B6, and 7H2 as a microheterogeneous peak (not shown) with a yield of 4 to 10 mg of IgG/mL ascites. The same elution position was observed for each of the five antibodies. Analyses of three of the rechromatographed MAbs under chromatofocusing conditions also showed one peak for 13H11 and 13G11 and a microheterogeneous peak for 6A6 with similar pH elutions for the three MAbs, 5.6, 5.7, and 5.8, respectively. In agreement with these results, the five MAbs were shown to belong to the same subclass, IgG1, κ light chain, by subtyping analysis. Analysis by SDS-PAGE of MAb 13G11 showed that purity of this MAb was >95% (Fig 2) with a major component on nonreducing gels (lane 1) and the heavy and light chains of the MAb, with contaminants <5% of protein visible under reducing conditions (lane 2). These results, in addition to the method used for hybridoma production (subcloning twice by limiting dilution), indicate that a true MAb was used in the assays described below. MAbs 6A6, 13H11, and 13G11 were analyzed for their ability to detect, by Western blotting, prekallikrein and/or kallikrein transferred from SDS-gels onto nitrocellulose membrane. They were also evaluated for their effect on prekallikrein activation or kallikrein activity in purified and plasma systems. Because these MAbs present similar characteristics in their recognition of both prekallikrein and kallikrein in the blotting assays and in inhibition of prekallikrein activation and kallikrein activity, one of them, 13G11, was selected for our studies.

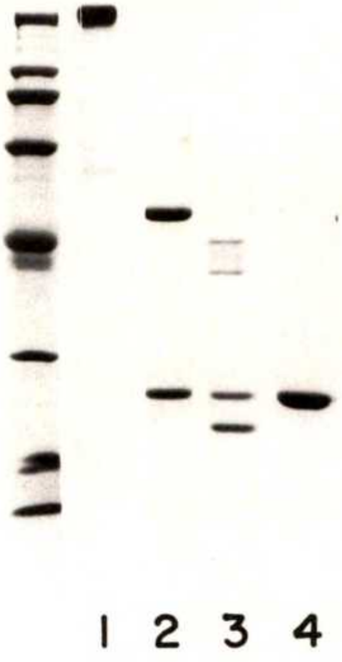

Fig 2. The pooled eluate fractions containing 13G11 (described in Fig 1) as well as the Fab fragments prepared from it were analyzed by SDS-gels. The electrophoregrams of 13G11 under nonreducing (~170 kd) and reducing conditions (~50 and 28 kd) are shown in lanes 1 and 2, respectively; those of Fab fragments under nonreducing and reducing (~28 kd) conditions are shown in lanes 3 and 4, respectively. Protein standards (200, 116, 93, 66, 45, 31, 22, and 14 kd) were assayed simultaneously (lane at left).

Under the conditions used for Fabs preparation by cleavage of 13G11 by papain (purified MAb and monitoring of its hydrolysis), only Fab and Fc fragments were produced. Therefore, on their separation either by ion-exchange or gel filtration chromatographies, the Fabs were obtained as a homogeneous species as analyzed by SDS-gels under reducing conditions (Fig 2, lane 4). The four species observed under nonreducing conditions (lane 3) were probably caused by formation of intradisulfide and interdisulfide bonds of cysteines not alkylated after cleavage of 13G11 by papain in the presence of mercaptoethanol.

Epitope location. MAb 13G11 bound to blots of both variants of nonreduced purified prekallikrein and kallikrein (Fig 3, lanes 2 and 3) at concentrations as low as 0.2 μg MAb/mL TBS-BSA. The stained blots were located at the same position of the stained bands on the SDS-gels of reduced purified prekallikrein (Fig 3, lane 1). When prekallikrein or kallikrein was reduced, reactivity was not observed at similar concentrations of antigen (shown for prekallikrein, Fig 3, lane 4). Transferring efficiency was not altered by reduction as assessed by staining of the SDS-gel after protein transfer and by Amido black staining of the proteins transferred onto the nitrocellulose membrane. Thus, the decreased reactivity was attributed to epitope alteration by reduction. An eightfold increase of the concentration of reduced kallikrein applied to an SDS-gel and then transferred to nitrocel-

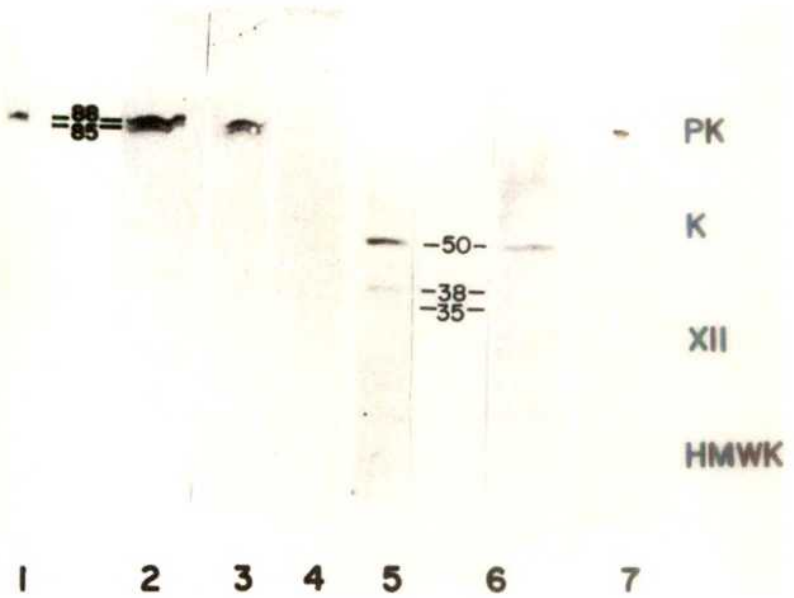

Fig 3. Western blots of prekallikrein (PK) and kallikrein (K) SDS-polyacrylamide gel electrophoregrams. Nonreduced prekallikrein (lane 2) and kallikrein (lane 3) or their reduced form (lanes 4 and 6, respectively), were probed with the MAb 13G11. Lanes 1 and 5 show the SDS-gel of reduced prekallikrein and kallikrein, respectively, after staining (Coomassie brilliant blue R250). In lane 7, prekallikrein, kallikrein, factor XII (XII) and HMWK (0.3 μg, 0.3 μL each) were applied directly to the nitrocellulose membrane for Western blotting. Prekallikrein or kallikrein (2.0 μg each) and 16 μg of kallikrein were used in the SDS-gels of blots of lanes 1, 2, 3, 4, 5, and 6, respectively. The indicated positions of prekallikrein and kallikrein (88 and 85 kd) and of kallikrein heavy (50 kd) and light (38 and 35 kd) chains were determined from the position of protein standards. Details are given in the Materials and Methods section.

lulose membrane allowed detection of the epitope on the heavy chain but not on the light chains (Fig 3, lane 6). Under these conditions, the heavy and light chains are well separated, as shown by the stained bands on the SDS-gels of reduced kallikrein (Fig 3, lane 5). Immunoblots with 13G11 of the SDS-gel electrophoregram of fresh normal plasma also show the presence of the epitope on the two prekallikrein variants (Fig 4, lane 2) with the same mobility as that of purified prekallikrein electrophoresed either in the absence (Fig 4, lane 1) or the presence of normal plasma (Fig 4, lane 3). As expected, the stained bands were not visible with the prekallikrein deficient plasma (Fig 4, lane 4) but were observed after supplementation of this plasma with prekallikrein (Fig 4, lane 5).

MAb 13G11 did not recognize the other proteins of the contact activation, factor XII and HMWK as judged either by direct application (Fig 3, lane 7) or by transfer from SDS-gels to the nitrocellulose membrane (not shown). In addition, the epitope was not present in the prekallikrein homologous proteins, factor XI and bovine plasma kallikrein,[12,32-35] as tested both by immunoblotting the proteins transferred from their SDS-gels and by ELISA (data not shown) under conditions that showed positive reaction with human prekallikrein. In agreement with these results, specific detection by 13G11 of the two prekallikrein variants was observed in the immunoblots of the normal plasma transferred from the SDS-gels (Fig 4, lane 2). These results also indicate that the two prekallikrein variants are not an artifact of protein purification.

Effect of MAb 13G11 on the activation of prekallikrein in plasma. These determinations were carried out either in prekallikrein-deficient plasma supplemented with purified prekallikrein or in normal plasma, in the presence or absence of 13G11, followed by their activation on kaolin. Using procoagulant assays, inhibition of activation of prekallikrein preincubated with the MAb and then supplemented to the prekallikrein-deficient plasma reached a maximum of ~60%

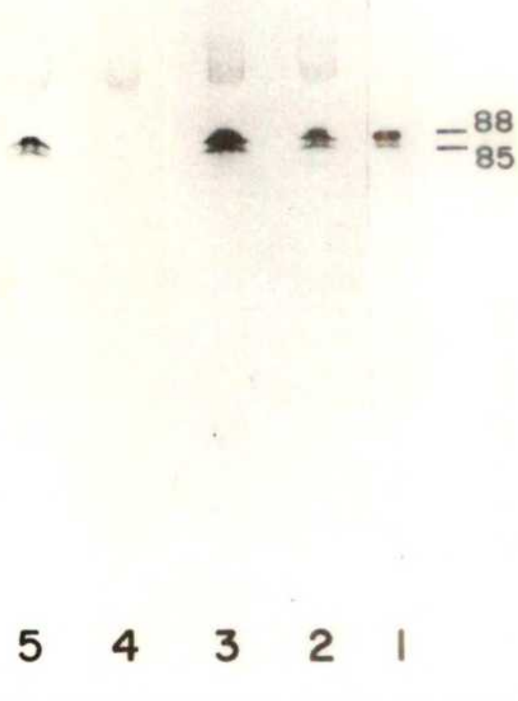

Fig 4. Western blots of plasma SDS-polyacrylamide gel electrophoregrams. Five microliters of normal plasma (lanes 2 and 3) or prekallikrein-deficient plasma (lanes 4 and 5) both with 5 μL (0.5 μg) of purified prekallikrein (lanes 3 and 5) were incubated for 10 minutes at 23°C and then subjected to SDS-gel and immunoblotting with MAb 13G11. For comparison, the same amount of purified prekallikrein was assayed (lane 1). Details are given in the Materials and Methods section.

at near equimolar concentrations of MAb/prekallikrein and remained independent of antibody concentration (Fig 5A). Inhibition (~60%) with a pattern similar to that for coagulation was observed when prekallikrein activation was measured by hydrolysis of a tripeptide chromogenic substrate for kallikrein after the MAb was incubated either with prekallikrein and then added to the prekallikrein-deficient plasma (Fig 5B) or with the normal plasma (Fig 5C). In agreement with the apparent identity of the prekallikrein purified and that present in plasma, as observed by immunoblotting assays, purification of prekallikrein did not alter its functional characteristics as assayed in a plasma environment. Inhibition of prekallikrein activation of normal plasma in the presence of Fabs of 13G11 was 87% of that observed in the presence of the entire MAb (Fig 6).

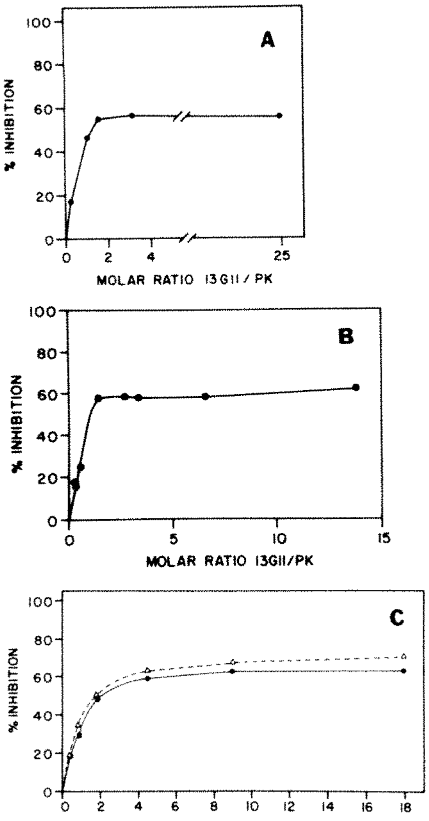

Fig 5. Inhibition by 13G11 of the procoagulant and amidolytic activities of prekallikrein in plasma. For determination of the effect of 13G11 on the procoagulant activity, prekallikrein (0.75 μg in 10 μL containing 3 mg of HSA/mL) was incubated with various amounts of MAbs (final volume 25 μL) at 37°C for 10 minutes before coagulation assay (A). For determination of the effect of 13G11 on the amidolytic activity, either prekallikrein (1.1 μg, 3.6 μL) mixed with prekallikrein-deficient plasma (13.4 μL) was incubated for 40 minutes at 37°C with various amounts of 13G11 (B) or incubation of the MAb was with 25 μL of normal plasma in a final volume of 45 μL (C) before being mixed with an equal volume of a kaolin suspension. Residual activity was determined with D-Pro-Phe-Arg-pNa (B) and with Bz-Pro-Phe-Arg-pNa (C). When normal plasma was used, experiments were also carried out under the same conditions except that the plasma (23.7 μL) was preincubated with 1.0 mol/L (1.3 μL) methylamine-HCl made in TBS, for 2 hours at 37°C (△- - -△). Details are given in the Materials and Methods section. Results expressed as percentage of inhibition were calculated on the basis of activity, in the absence of antibody, as 100% (or 0% inhibition). Results were plotted against the molar ratio of MAb 13G11/prekallikrein.

Inhibition of prekallikrein activation, in plasma, determined both by coagulant activity and hydrolysis of synthetic substrates never reached 100%. To ascertain whether this was caused, in part, by inaccessibility of trapped kallikrein by α_2-macroglobulin,[36,37] normal plasma was preincubated with methylamine[38] to destroy the inhibitory activity of α_2-macroglobulin prior to incubation with the MAb. Treatment of plasma with methylamine did not alter activity of the control (MAb absent, 100% activity) as shown by the observation of a similar inhibition both in methylamine treated and nontreated plasma (Fig 5C).

Do 13G11 and HMWK compete for interaction with prekallikrein or kallikrein? Optimal hydrolysis of HMWK requires the presence of kallikrein heavy chains.[13,19] Because this chain also contains the epitope for 13G11, we tested whether 13G11 interacted with the sites on kallikrein and prekallikrein required for HMWK binding using functional assays for kallikrein and competition assays for prekallikrein. The rate of cleavage of HMWK by kallikrein, analyzed by SDS-gels, was similar when the assays were carried out in the presence of MAb to factor XI (control antibody) or of MAb 13G11 (Fig 7). The results suggest that different sites exist on the kallikrein heavy chain for binding of MAb 13G11 and HMWK. The competition of HMWK with 13G11 for binding to prekallikrein coated on microtiter plates was tested using a 5-, 10-, or 100-molar excess of HMWK over MAb. The increase in HMWK concentrations did not decrease binding of 13G11 to prekallikrein (Table 1). Thus, 13G11 does not recognize the region required for binding of prekallikrein to HMWK. Because similar high-affinity binding between HMWK and prekallikrein or kallikrein heavy chains with a resultant formation of 1:1 complexes has been reported,[39] the same region in both enzyme and zymogen must be involved in the binding to HMWK. Therefore, the two independent approaches used indicate that the mechanism of inhibition of prekallikrein coagulant

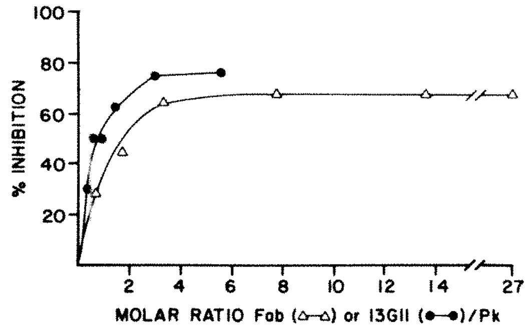

Fig 6. Inhibition of plasma activation by Fab fragments of 13G11. Normal plasma (25 μL) was incubated with several amounts of Fabs (△—△), followed by treatment with kaolin and assay with D-Pro-Phe-Arg-pNa as described in the legend to Fig 5. For comparison, parallel assays were carried out with the entire MAb (●—●) instead of the fragments. Expression of results is given in the legend to Fig 5.

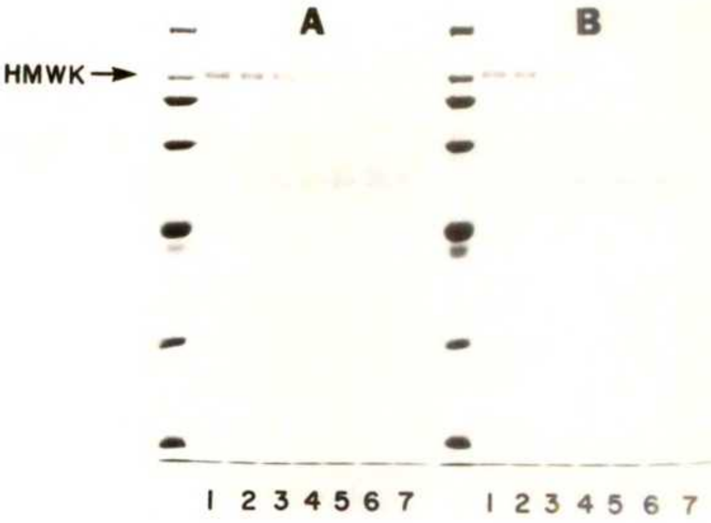

Fig 7. Hydrolysis of HMWK by kallikrein in the presence of 13G11. Kallikrein was incubated with either MAb to factor XI (A) or 13G11 (B), followed by addition of HMWK. Each mixture (A or B) was then further incubated several times; after these incubation periods, samples were removed for SDS-gel analysis (reducing conditions), lanes 1, 2, 3, 4, 5, 6, and 7, respectively. For comparison of the size of the HMWK-cleaved polypeptides, two samples of the protein standard mixture were also electrophoresed. The position of uncleaved HMWK is indicated by an arrow. Details are given in the Materials and Methods section.

activity is not caused by interaction of 13G11 with the binding site on prekallikrein or kallikrein for HMWK.

Effect of MAb 13G11 on kallikrein activity and prekallikrein activation in a system mimicking contact activation. For a better understanding of the effect of 13G11 on protein interaction during contact activation in plasma, we used a purified system which could mimic that of contact activation. For testing whether the inhibition by 13G11 was at the level of prekallikrein activation or kallikrein activity, 13G11 was incubated either with purified prekallikrein, HMWK, factor XII, and kaolin or with the same mixture after incubation in which kallikrein is formed. The inhibition of ~80% observed on the activation of prekallikrein by kaolin-activated factor XII in the presence of a 1- to 9-molar excess of 13G11 over prekallikrein (Fig 8A, ○—○) was marked higher than that of 55% to 25% observed both when the assays were carried out in the absence of HMWK (Fig 8A, △—△) or when 13G11 was added after kallikrein formation (Fig 8A, ●—●). Kallikrein activity generated from prekallikrein by kaolin-activated factor XIIa in the absence of MAb (~1.5 μmol *p*-nitroaniline formed/min/mL) was not dependent on HMWK. The unusual inhibition pattern of both prekallikrein activation in the absence of HMWK (Fig 8A, △—△) and kallikrein activity (Fig 8A, ●—●) was similar to that observed when prekallikrein preincubated with 13G11 was activated by factor XII fragments (data not shown). The results indicate that under these conditions inhibition is probably at the level of kallikrein activity.

Table 1. HMWK Does Not Compete With MAb for Binding to Prekallikrein Coating Microtiter Plates

Prekallikrein μg/Well	HMWK μg/Well	Absorbance at 405 nm
0	0	0.07
0.2	0	1.47
0.2	2.0	1.46
0.2	4.0	1.45
0.2	40.0	1.47

For this determination, prekallikrein was bound to microtiter plates. Binding of MAb (0.4 μg) was in the presence of HMWK as indicated. ELISA assays were carried out as described in the Materials and Methods section.

The mechanism of kallikrein inhibition, in contrast to that observed for surface activation of prekallikrein in plasma, was likely caused by steric hindrance, since no inhibition of kallikrein amidolytic activity was observed when Fab fragments of 13G11 were used instead of the entire IgG (data not shown). Moreover, an inhibition profile similar to that observed with kallikrein was observed when the prekallikrein activation by 13G11 was accomplished in the absence of HMWK (Fig 8A). Thus, the inhibition in plasma was not caused by interference of 13G11 on formation of the complex prekallikrein–HMWK. Under the conditions used in the system mimicking contact activation, autoactivation of factor XII (not detectable) and trace contaminating proteins in purified prekallikrein and HMWK (each <5% of total activity) were not expected to affect the results.

Comparison of the results observed with plasma and those with the system mimicking contact activation indicates that inhibition of plasma activation (HMWK is also present in prekallikrein-deficient plasma) are at the level of prekallikrein activation. Because our results also indicate that 13G11 interacts with prekallikrein at a site different from that containing the HMWK binding region, the effect of 13G11 on activation of prekallikrein, in the presence of HMWK, by factor XIIa prebound to kaolin, was investigated. Similarly to the results observed with plasma (Fig 5), inhibition was 67% at near equimolar concentrations of 13G11/prekallikrein and remained constant at high concentrations of the antibody (Fig 8B). When a MAb to factor XI was used instead of 13G11, the activation of prekallikrein was not inhibited. The results suggest a significant role for the region of prekallikrein exhibiting the epitope for 13G11 on prekallikrein activation by factor XIIa bound to a surface.

DISCUSSION

MAbs 6A6, 13H11, and 13G11 show similar characteristics apparently suitable for structural and functional studies.

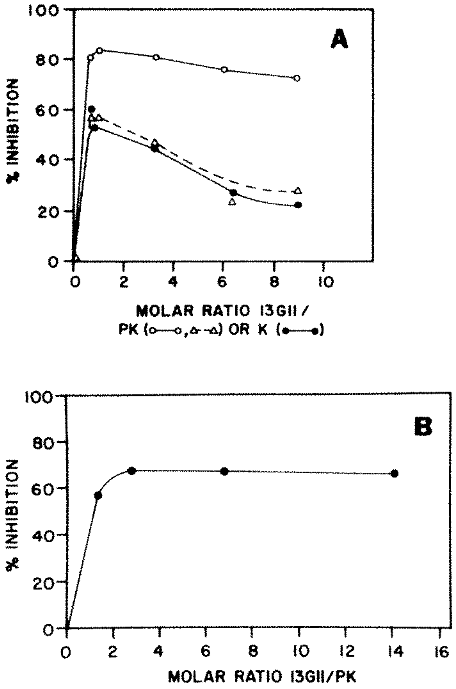

Fig 8. Effect of MAb 13G11 on prekallikrein activation by kaolin-activated factor XII, with or without HMWK and on kallikrein activity with HMWK. The effect of 13G11 on prekallikrein activation, in the mimic contact activation system, tested in two ways, are represented in A and B. In A, the effect of MAb on prekallikrein activation without HMWK and on kallikrein activity were also tested. For A, each assay mixture contained prekallikrein, HMWK, kaolin suspension, and various amounts of MAb. The reaction started by addition of factor XII followed by incubation and determination of protease activity with D-Pro-Phe-Arg-pNa (O——O). Assays were also carried out under the same conditions except that HMWK was absent (△- - -△). For testing the effect of MAb 13G11 on kallikrein activity, prekallikrein was activated before MAb addition in a system as described above except that MAb was absent. After incubation, MAb was added followed by further incubation for 40 minutes and determination of residual activity (●——●). For B, each incubation mixture containing prekallikrein (0.36 μg), HMWK (2.9 μg), and various amounts of 13G11 in a final volume of 28 μL were incubated for 40 minutes at 37°C. The mixture was then mixed with a kaolin suspension (15 μL) containing the factor XIIa for 5 seconds and incubated at 23°C for 55 seconds. An aliquot (15 μL) was then withdrawn for aminolytic activity determination with D-Pro-Phe-Arg-pNa. Details are given in the Materials and Methods section. Expression of the results is given in the legend to Fig 5.

Detailed analyses by Western blots showed that MAb 13G11 binds to prekallikrein whether applied directly to the nitrocellulose membrane or after transfer from SDS-gels (Fig 3). Hence, either the presence of SDS did not destroy epitope topography or enough of the SDS was removed during transfer and washing of the nitrocellulose membrane to allow epitope to be reformed. Because the epitope was also detectable on kallikrein, cleavage of prekallikrein to form the active-site pocket did not affect epitope availability. That the antibodies recognize both variants of prekallikrein and kallikrein (Fig 3) indicates that the epitope is not located on the region of the molecule missing in the 85-kd prekallikrein, probably a carbohydrate.[1] When prekallikrein or kallikrein was reduced, however, it was difficult to see epitope reaction. Apparently, disulfide bridges are involved in either the formation or exposure of the epitope. Because an eight-fold increase in kallikrein concentration allowed epitope detection on its heavy chain, a possible explanation for the decrease of epitope concentration was scrambling of the disulfide bridge(s) during reduction and reformation.

MAb 13G11 appears specific for prekallikrein in plasma (Fig 4) and, since it did not show any reactivity with factor XII or HMWK, it was useful for studies of protein interactions during the contact activation reactions. MAb 13G11 did not inhibit cleavage of HMWK by kallikrein (Fig 7) and HMWK did not compete with 13G11 for binding to prekallikrein (Table 1). These findings indicate that this MAb binds to prekallikrein and kallikrein at a site different from the domain responsible for the binding of HMWK. Therefore, although prekallikrein circulates in plasma associated with HMWK, under these conditions the epitope must be available to access by the antibody. Because the epitope was not detected on human factor XI or bovine plasma kallikrein, it is likely part of a region of prekallikrein with high antigenicity and evolutionary variability.[40,41]

Although MAb 13G11 recognizes an epitope located on the heavy chain (Fig 3), it also partially inhibits catalytic activity toward tripeptide *p*-nitroanilide substrates (Fig 8A), an exclusive property of the light chain.[13] Because kallikrein activity was not affected by Fab fragments of the 13G11, this inhibitory activity probably is due to steric hindrance. Thus, although the epitope is on the heavy chain, it seems to be spatially located near the active site on the light chain. Studies with the purified system mimicking contact activation show that the presence of HMWK has an important effect on the ability of the antibody to impair surface activation of prekallikrein. Thus, formation of prekallikrein–HMWK or prekallikrein–HMWK–kaolin complexes may modify the epitope–active site topography. Published reports suggest a conformation change of factor XII[42] and kallikrein[19] on their binding to a surface. Therefore, HMWK conformation change on its binding to a surface would not be unexpected. A conformation change of prekallikrein on its binding to HMWK may also occur.

Inhibition of prekallikrein activation by 13G11 both in plasma and purified systems never reached 100%. A similar partial inhibition by a MAb that recognizes the heavy chain of factor XI has been reported before.[43] The reason for a partial inhibition by MAbs that recognize the noncatalytic chain is not now clear.

The comparable pattern of inhibition by 13G11 observed with purified prekallikrein both in a purified system mimicking contact activation and in its plasma environment shows that the effect of the MAb observed in normal plasma was on specific interactions in the contact activation system. With the purified system mimicking contact activation, we observed that inhibition of kallikrein as well as that of prekallikrein in the absence of HMWK has a tendency to decrease with higher concentrations of MAb (Fig 8A). The decreased inhibition at higher concentrations of MAb does not seem to be caused by decreased binding of MAb, since Western blots were equally visible at MAb concentrations from 0.2 to 2 μg/mL TSB-BSA. This unusual inhibition pattern was not caused by the presence of contaminant MAbs or kallikrein inhibitors. MAb 13G11 was established as a true MAb by two criteria, its production by limited dilution and its characterization as a homogeneous protein by ion-exchange chromatography and chromatofocusing. The presence of a hypothetic kallikrein inhibitor was not detected by SDS-PAGE analysis (Fig 2). Hence, the cause of this unusual inhibition pattern requires further investigation.

The observed inhibition of prekallikrein activation (HMWK present), measured by D-Pro-Phe-Arg-PNa hydrolysis, was the result of two separate effects of MAb 13G11. The antibody partially inhibits prekallikrein activation by surface-activated factor XIIa. In addition, MAb 13G11 partially inhibits the activity of the kallikrein formed on activation by factor XIIa of the residual prekallikrein that escaped inhibition by the antibody. Because inhibition of kallikrein activity was only 20% to 40% (Fig 8A), the inhibition by 13G11 on the kallikrein formed on activation of the prekallikrein incubated with 13G11 represents a small contribution in the total measured inhibition of prekallikrein activation. Because no inhibition was observed on the kallikrein activity by 13G11 fragments (results not shown), the 13% smaller inhibitory effect on prekallikrein activation by Fab fragment as compared with that by the intact MAb, measured by hydrolysis of the chromogenic substrate (Fig 6) is probably explained by the lack of inhibition at the level of kallikrein activity in the presence of the 13G11 fragment. Therefore, most of the inhibition measured following activation of the prekallikrein preincubated with 13G11 is at the level of prekallikrein activation and not at the level of kallikrein activity. This inhibition was not caused by competition of 13G11 with HMWK for the same sites on prekallikrein with subsequent dissociation of the prekallikrein–HMWK complex. MAb 13G11 appears to bind to or near a region of prekallikrein involved in its activation by factor XIIa. Indeed, activation of prekallikrein, in the presence of HMWK, by factor XIIa previously bound to the kaolin was inhibited by 13G11 in a fashion similar to that observed for plasma and in the purified system mimicking contact activation in which factor XII, prekallikrein, HMWK, kaolin, and 13G11 were incubated simultaneously. Because only partial inhibition on prekallikrein activation has been observed, binding of 13G11 to prekallikrein might decrease the access of factor XIIa to its binding or cleavage sites on prekallikrein. Because the 13G11 fragments did not seem to affect prekallikrein activation in plasma, the region of the prekallikrein recognized by 13G11 may partially overlap or be located in very close proximity to the region recognized by factor XIIa. Binding of 13G11 (or its fragments) will limit the access or the magnitude of binding of factor XIIa to the prekallikrein site.

This monoclonal antibody should have further use in assays of both function and antigen concentrations in plasma samples. The ability of this antibody to detect, by Western blotting, prekallikrein in plasma and kallikrein and kallikrein heavy chains in purified systems should allow recognition of formation of the heavy chain of kallikrein or of its products, such as fragments and kallikrein–inhibitor complexes, in pathophysiologic conditions.

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A Two-Color Flow Cytometry Assay for Detection of Hairy Cells Using Monoclonal Antibodies

By Jørgen Schøler Kristensen, Jørgen Ellegaard, and Peter Hokland

We have developed a simple two-color immunofluorescence assay equally suited for microscopy and flow cytometry detecting hairy cells (HCs) in single cell suspensions, based on the concomitant reactivities with the B cell-specific monoclonal antibody B1 (CD20) and the monocyte/HC-associated antibody SHCL-3 (CD11c). Thus, HCs can be demonstrated in peripheral blood, bone marrow, and spleen specimens from hairy cell leukemia (HCL) patients even when they constitute <1% of the cell suspension. Likewise, admixture experiments with normal mononuclear cells and the MOLT-4 T-acute lymphocytic leukemia (ALL) cell line demonstrated that HCs could be detected in amounts as low as 1%. The validity of this assay has been ascertained by the lack of double marker positivity in cell suspensions from B-chronic lymphocytic leukemia (CLL) and acute myelogenous leukemia (AML) patients that only expressed B1 or SHCL-3, respectively. Furthermore, other malignant blood diseases, including malignant lymphomas, acute leukemias, and chronic leukemias disclosed no double marker positive cells. In a clinical setting, this assay was used for purifying HCs (by flow cytometry) from the peripheral blood from patients with no apparent morphological evidence of circulating HC infiltration and for monitoring the effect of interferon therapy. In conclusion, this assay should be of value for both diagnosis and monitoring patients with HCL.

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HAIRY CELL leukemia (HCL) is recognized to be of B cell origin due to the presence of B cell-restricted surface determinants[1-3] and by the rearrangement of immunoglobulin genes in HCs.[4] Furthermore, based on the reactivity of HCs with plasma cell-associated antibody PCA-1, HCL has been suggested to be a pre-plasma cell tumor.[5] In line with this observation, recent evidence using B cell differentiation stimuli has suggested that the normal counterpart of HCs might be an activated B-lymphoblast.[6,7] On the other hand, HCs can express antigens normally associated with myelomonocytic cells, especially the Mo1 antigen, which has been found on most but not all HCL samples.[2,3,8,9] The functional significance of the presence of these myeloid antigens on a purported B cell tumor is at present unknown.

Because HCL is a well-established clinicopathologic entity, its diagnosis and longitudinal monitoring of the disease have proven difficult, partly because the number of circulating HCs can be low and partly because the morphology and tartrate-resistant acid phosphatase (TRAP) staining are not necessarily reliable diagnostic parameters.[10] Furthermore, since none of the monoclonal antibodies (Mabs) yet published have shown specificity for HCs, immunologic identification of these cells is based on the successive reactivities with different Mabs.[9] Because no other hematopoietic cells to our knowledge have shown similar evidence of simultaneous expression of myelomonocytic and B cell-associated antigens, we hypothesized that an assay identifying both such differentiation antigens concomitantly on the single cell level might be an optimal way of identifying HCs. Here we present data on such a test using the B cell-specific B1 antibody and the monocytic SHCL-3 antibody. HCs could be definitively identified and purified even when they constituted <1% of cell suspensions.

MATERIALS AND METHODS

Patients. Samples from 18 patients with clinically and morphologically verified HCL were obtained after informed consent was given. No patients had received cytostatic treatment prior to sampling.

Isolation of mononuclear cells. Mononuclear cells (MNCs) were obtained from peripheral blood by Ficoll-Hypaque density centrifugation. MNC samples from spleens were obtained after mincing freshly obtained tissue with forceps and scissors, followed by repeated suction in a 21-gauge needle. Samples were either used fresh or were cryopreserved in the vapor phase of liquid nitrogen in 10% dimethylsulfoxide (DMSO) and 20% fetal calf serum (FCS) The MOLT-4 T-ALL cell line was grown in 75-cm^2 culture flasks in RPMI 1640 medium supplemented with 10% FCS and antibiotics.

Immunofluorescence staining. One million cells in 100 μL were incubated with approximately diluted [in phosphate-buffered saline (PBS) with 2.5% human AB-serum (PBS-AB)] ascites containing the Mabs at 4°C for 30 minutes. Reactivities and sources of the antibodies used are shown in Table 1 After two washes in PBS-AB, the cells were incubated with a fluorescein (FITC)-conjugated $F(ab)'_2$ rabbit anti-mouse IgG (R/M-Ig FITC, Dakopatts, Copenhagen). After a 30-minute incubation period at 4°C, the cells were washed three times in PBS-AB and analyzed as described below.

Two-color fluorescence labeling of HCL samples. One million cells were labeled consecutively with SHCL-3 in its unconjugated form, followed by R/M-Ig FITC as described above Immediately thereafter, the cells were incubated with biotin-conjugated anti-B1[11] (Coulter Immunology, Hialeah, FL) and phycoerythrin (PE)-conjugated avidin (dilution 1:40, Becton Dickinson, Mountain View, CA). All incubations were performed at 4°C for 30 minutes followed by three washes in PBS-AB. In this manner, B1 antibody binding sites were labeled bright red, whereas the SHCL-3 antibody binding sites were labeled green. Negative controls for the SHCL-3 Mabs included an IgG_{2a} antibody with no known reactivity (8A7) and for B1 a biotin-conjugated CD8 Mab (Leu2a, Becton Dickinson).

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Table 1. Monoclonal Antibodies Used in This Study

CD*	Mab Used	Reactivity	Source
3	UCHT-1	Mature T cells	B. Perussia
10	J5	CALLA	J. Ritz
11b	MO1	C3bi receptor	J. D. Griffin
13	My7	Immature myeloid	J. D. Griffin
14	My4, Mo2	Monocytes	J. D. Griffin
11c	SHCL-3	LFA,† gp 150/95	R. Schwarting
19	B4	B cells	L. M. Nadler
20	B1	B cells	L. M Nadler
21	B2	C3d receptor	L. M. Nadler
33	My9	Immature myeloid	J. D. Griffin
None	Ia	HLA-DR antigen	L. M Nadler

*Cluster of differentiation defined by the International Leucocyte Typing Workshops (Oxford revision, 1986).

†Leucocyte Function Antigen. (For a thorough review of these reagents, see A. McMichael, et al, eds: Leucocyte Typing Workshop III, Oxford University Press, 1986.)

Normal rabbit serum conjugated with FITC was used as a second-layer control (Dakopatts).

Flow cytometry analysis. Antibody-labeled cells were analyzed by flow cytometry in an EPICS C flow cytometer (FCM) (Coulter) after appropriate forward-lightscatter gating. For each sample, at least 10,000 cells were analyzed using the log amplification mode, with negative controls being MNCs labeled with second antibody layer alone. The intensity of cell surface fluorescence was estimated by the mean channel number of the log-fluorescence histogram. Double-labeled cell suspensions were analyzed after appropriate forward-lightscatter gating and optimization of the filters to PE and FITC using the two-parameter histogram. The number of double-labeled cells was calculated using the QuadStat statistics program of the EPICS C. For optimization purposes, the flow cytometer was adjusted daily using green and red fluorescent calibration beads (Coulter Electronics, Luton, England).

RESULTS

Reactivity of HCs with various myeloid and lymphoid antibodies. In the process of phenotyping blood, bone marrow, and spleen specimens from HCL patients we noticed a marked heterogeneity in the expression of most of the leukocyte differentiation antigens detected by Mabs both with respect to the number of positive cells and to the intensity of the antigens detected. As is shown in Table 2, the reactivity with an antibody against a myeloid differentiation antigen (eg, My7) on homogenous HCL spleen suspensions could vary between 1% and 86% of positive cells. The same was true for the My8, My9, Mol, and Mo2 antigens. In contrast, the monocytic antibody My4 consistently reacted with most of the HCs, albeit with low intensity in some instances. The SHCL-3 antibody was present in high density on all HC specimens tested. Whereas the expression of myeloid antigens varied from patient to patient, the expression of most B cell differentiation antigens detected in this study was consistently high (eg, J5, B1, and B4). Indeed, only the B2 antigen, which detects the C3d receptor,[12] was present in low numbers. Consistent with other studies, we found that almost all HCs expressed only one immunoglobulin light chain, thus demonstrating the monoclonality and B cell derivation of the malignant cells. Concerning the density of these B cell-associated antigens, it is noteworthy that whereas the common ALL antigen (CALLA) (J5), B4, and light chains were only slightly expressed, the B1 antigen was always present in high density.

Table 2. Analysis of Surface Marker Antigens on HC Suspensions

Mab	Spleen 1	 2	 3	 4	 5	 6
My4	90*	90	76	72	56	54
My7	<1	81	86	17	15	26
MY8	<1	<5	17	<5	<5	<5
My9	83	14	14	23	5	21
MO-1	64	9	26	40	34	49
MO-2	21	75	43	<2	<2	7
J5	82	87	81	10	11	53
B1	91	>95	>95	61	72	52
B2	12	16	11	<2	11	<5
B4	78	>95	27	80	76	63
Ia	90	95	95	80	90	67
UCHT1	8	3	2	20	2	26
SHCL-3	80	92	89	63	85	74
SHCL-3/B1	91	95	94	56	74	58
B1-BIO	76	81	85	69	68	54
κ/λ	κ†	λ	λ	λ	κ	λ

*Percentage of positive cells

†Dominating light chain

Two-color fluorescence detection of HCs using SHCL-3 and B1. Although both SHCL-3 and B1 are highly expressed on HCs, their expression on monocytes and B cells, respectively, make their use as single reagents for the detection of HCs impossible. With the introduction of PE–avidin conjugates and the availability of biotin-conjugated Mabs, however, it has become possible to perform two-color studies both with the fluorescence microscope as well as with single-laser flow cytometers since FITC and PE have different emission spectra but share a common excitation wavelength (488 nm). Because both B1 and SHCL-3 react with HCs, we designed a dual-color fluorescence test using SHCL-3+R/M-Ig FITC and B1-biotin + avidin-PE. In the fluorescence

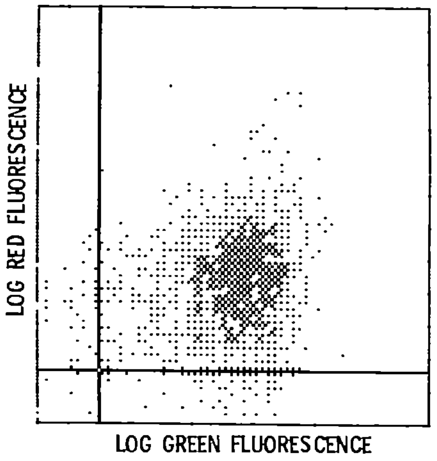

Fig 1. Hairy cells from peripheral blood with 71% TRAP+ cells identified by two-color flow cytometry. Two parameter dot plot of integrated SHCL-3 (*x*-axis) B1 (*y*-axis) signals analysed by Quadstat statistics showed 61% double-marker–positive cells. Negative controls revealed 0.5% to 1.3% double-marker–positive cells.

Table 3. Analysis on Non-HCL Samples

Source	Mab B1	 SHCL-3	 SHCL-3/B1
Normal donor MNCs (25)	5–14*	7–18	0.1–1.1 (0.5%)‡
B-CLL (12)	45–91	1–3	0.2–0.9
AMML (5)	2–7	11–76	0.2–0.5
B lymphoma (18)	33–78	2–8	0.1–1 0 (0.7%)
CML (3)	1–4	2–7	0.1–0.8
C-ALL (4)	1–83	0–4	0.3–0.7
T-ALL (1)	1	1	0 1
ITP† (2)	8–14	8–19	0.8–1.2
AIHA‡ (2)	5–11	6–13	0.9–1.0
HCL (18)	1–82	2–92	0 1–83

Immune thrombocytopenia

*Range of percentage of positive cells in cell suspensions from peripheral blood.

†Autoimmune hemolytic anaemia; ‡Percentage of positive cells (mean).

microscope, both double-marker–positive cells (with a tendency for forming aggregates) and scattered cells expressing only one of the antigens could be clearly identified. Moreover, as is shown in Fig 1, the majority of HC spleen suspensions were SHCL-3/B1-positive, and in EPICS C flow cytometer double-marker–positive cells could easily be demonstrated. As compared with the microscope reading, the population of SHCL-3+/B1– cells was higher, and the proportion of double-marker–positive cells was somewhat lower. The reason for this discrepancy is undoubtedly the aggregation of HCs by B1, which could already be observed in the microscope after incubation with anti-B1 antibody. Because FCM automatically excludes larger cell aggregates from analysis, our assay slightly overestimates the number of SHCL-3+/B1– cells and slightly underestimates (100% *v* 92%) double-marker–positive cells.

A series of experiments was performed to validate this assay. Thus, negative controls including relevant second-layer antibodies only and first-layer Mabs of similar isotype with no known specificity all gave <1% reactivity. Because B1 and SHCL-3 are expressed in a number of malignant blood diseases, such specimens represented particularly well-suited controls. Thus, as is shown in Table 3, cells from five patients with acute myelomonocytic leukemia (AMML), and from 12 with chronic lymphocytic leukemia (CLL) that expressed one of the antigens were negative (Fig 2A through C). Moreover, cells from 18 patients with B1+ leukemic non-Hodgkin's lymphoma were also analyzed and likewise showed few (0.1% to 1.0%) double-marker–positive cells. From these data, we conclude that this method unequivocally identifies HCs with no demonstrable positivity on other malignant blood cells.

In an attempt to evaluate the sensitivity of the assay, we added homogenous HCs from spleen tissue (>95% pure) to the T-ALL cell line MOLT-4 and to peripheral blood MNCs. Another admixture experiment using peripheral blood HCs from a HCL patient (49% SHCL-3/B1 positive) was performed to detect differences between the detection of HCs from spleen tissue and HCs from peripheral blood. The results shown in Table 4 and Fig 3 show that double-marker–positive cells in numbers very close to those expected were observed. Such an admixture experiment can, however, only represent an idealized situation in which double-marker–positive cells are identified in a known population of negative cells. To evaluate the assay in HCL patients with possible residual disease, we investigated a larger group of patients in whom double-marker–positive cells were present in percentages varying from 0.5% to 5%. From a patient with 1.2% SHCL-3/B1 positive cells in the peripheral blood MNC suspension (in which morphology did not reveal any HCs), we purified the double-marker–positive cells using FACS on the FCM. As shown in Fig 4, sorted cells (when reanalyzed) were 94% double-marker–positive and on Giemsa-stained smears had the morphology of HCs. Together, the data in Figs 3 and 4 show that the SHCL-3/B1 assay is capable of identifying very small numbers of circulating HCs when morphology and cytochemistry have failed.

When the number of SHCL-3/B1-positive cells was compared with TRAP staining in 18 samples from nine patients, a surprising heterogeneity of reactivity patterns was observed (Fig 5). Thus, although some of the samples showed a close correlation between the double-marker assay and TRAP, two patients were 60% to 82% SHCL-3/B1 positive but TRAP negative. When evaluated on Giemsa-stained smears, one of the patients had >60% circulating HCs, the other patient showed only abnormal MNCs with some indications of HCs. Diagnosis was made on bone core biopsis. Thus, these patients demonstrate the value of the immunologic assay when TRAP staining is negative or when the HC morphology is atypical.

To elucidate the clinical value of this assay further, we

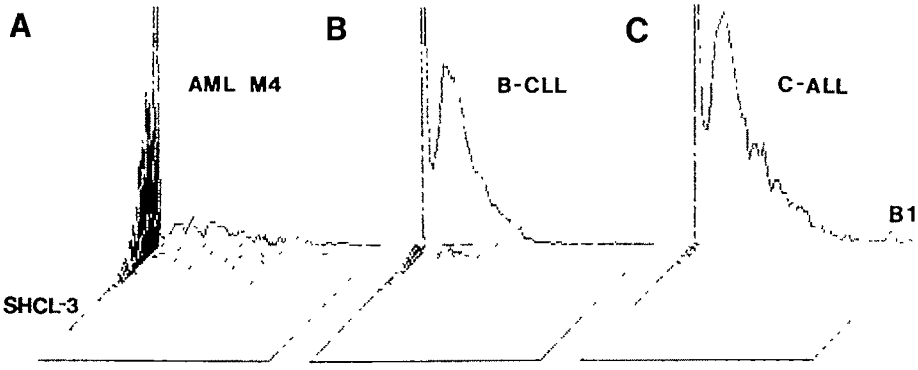

Fig 2. Three-dimensional histograms showing the results of double-labeling cases of: (a) SHCL-3+ AML, (b) B1+ CLL, (c) B1+ ALL. In all cases, <1% double-marker–positive cells were identified.

Table 4. Effect of Adding HCS to Double-Marker–Negative Cell Suspensions

Source	0%**	1%	5%	10%	25%	50%
MOLT-4*	13/0†	82/100	ND	921/1,000	ND	4,878/5,000
PB-MNC‡	11/0	917/1,000	4,413/5,000	9,192/10,000	23,897/25,000	ND
PB-MNC§	14/0	47/50	ND	431/500	ND	2,349/2,500

ND, not done.

*Addition of graded numbers of splenic HCs (>95% SHCL-3/B1+) to the T-ALL cell line MOLT-4 (CD3 and 7+, CD11c and 20−).

**Final percentage of HCs in suspension.

†Observed/expected number of SHCL-3/B1+ cells.

‡Addition of graded numbers of splenic HCs (>95% SHCL-3/B1+) to peripheral blood MNCs from a normal donor with 0.01% SHCL-3/B1+ cells.

§Addition of graded numbers of peripheral blood HCs (49.7% SHCL-3/B1+) to peripheral blood MNCs from a person with 0.01% SHCL-3/B1+ cells. Calculation was performed using the same criteria (quadrant setting) as in the control experiment

tested ten HCL patients receiving treatment with α-interferon (α-IFN) (2×10^6 U/m^2 three times a week) longitudinally for 3 to 9 months. Figure 6 shows that most patients exhibited a gradual decline in the circulating number of HCs after an unexplained rise on treatment day 1. This decrease in double-marker–positive cells correlated closely with a favorable clinical response to IFN treatment (B. Nielsen, manuscript in preparation). From these data, we conclude that the SHCL-3/B1 assay can be valuable in evaluating treatment response of patients with HCL.

DISCUSSION

The diagnosis of HCL is usually based on the demonstration of HCs—often in a spleen specimen—with the well-known morphology and TRAP positivity, but alternatively the diagnosis can be made by bone marrow core biopsy even in the absence of circulating HCs.[13] In addition, cells positive for B cell antigens with a predominantly mature immunologic phenotype are considered strong supportive evidence. No single surface antigen has, however, been found specific for HCs. That HCs express myelomonocytic antigens (Tables 1 and 2) distinguishes this disorder from any other malignant blood disease of B cell origin. Thus, very few, if any, pre-B-ALL, B-CLL or B lymphoma cells express antigens on the Mo− or My− series,[14,15] let alone the SHCL-3 antigen that, apart from HCL, is expressed exclusively on normal and malignant myeloid cells. Some controversy has existed concerning the reactivity of HCs with myeloid Mabs, especially the Mo1(CD11b),[2,3,9] but the data shown in Table 2 clearly show that CD11b antigen reactivity on HCs varies to a large extent from patient to patient. The presence of these myelomonocytic antigens is puzzling, especially in light of the recent demonstration of immunoglobulin gene rearrangements[4] and the secretion of monoclonal immunoglobulin in HCs.[5] HCs, however, are capable of phagocytosis and lysozyme discharge, as evaluated by electron microscopy[16,17] and, in a recent study, we showed functional phagocytic characteristics of HCs.[18] Although the B2 antigen contains both the C3d receptor[19] as well as the receptor for Epstein-Barr virus (EBV),[20] no biologic function has been attributed to the other B cell-related antigens used in this study. The presence of the B1, B2, and B4 antigens on HCs demonstrated here and the reactivity with the plasma cell-associated PCA-1 antigen[5] clearly indicate that the maturational stage of HCs lies between the antigen-activated B lymphoblast and the pre-plasma cell.

In this report, we have shown that the concomitant reactivity of two monoclonal antibodies directed against antigens on different hematopoietic cell lineages enables identification of HCs in cell suspensions from peripheral blood, spleen tissue, and bone marrow. The assay is, however, at a disadvantage in the rare HCL cases without circulating HCs[13] and in those in which suitable cell suspensions from the bone marrow can not be obtained. The double-marker assay was optimized by choosing antibodies that had shown the most consistent reactivity and noticeable high density on HCs and by using admixture experiments and FACS to verify the capacity of

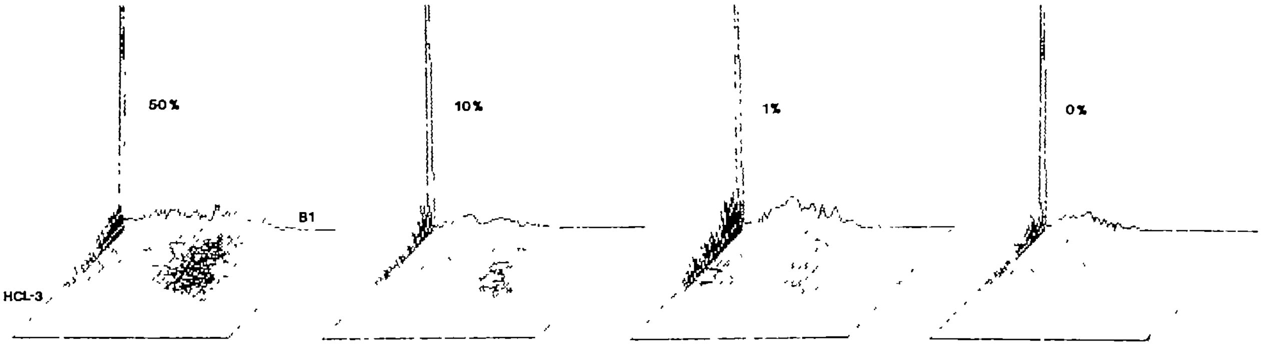

Fig 3. Result of adding double-marker–positive HCs (>95% B1/SHCL-3) to the MOLT-4 T-ALL cell line. Even addition of 1% HCs is identifiable. Quadstat analysis of the mixtures showed 48.7%, 9.2%, 0.8%, and 0.0% double-marker–positive cells, respectively. Discrimination between negative and positive cells was unaltered on Quadstat analysis throughout the experiment.

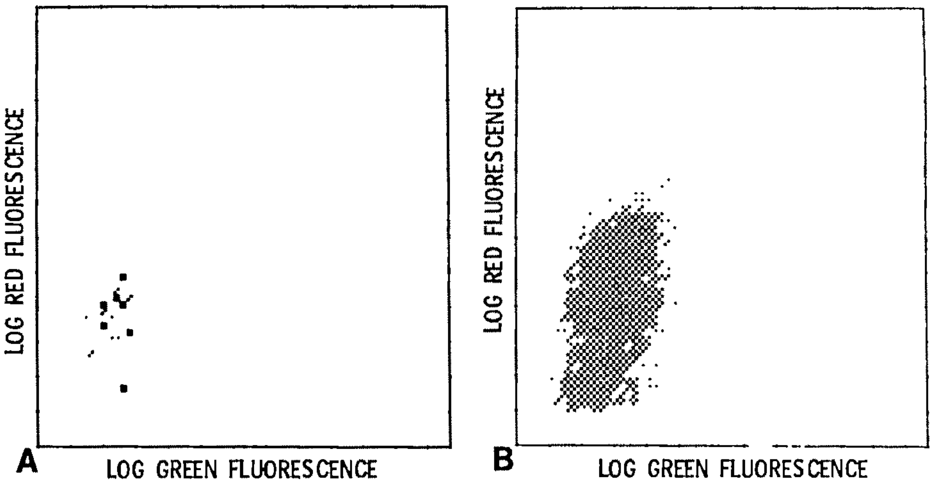

Fig 4. (A) MNC cell suspension containing 1.2% SHCL-3/B1-positive cells from a patient with HCL. Negative controls with irrelevant first layers contained 0.1% positive cells. (B) After sorting on a flow cytometer, reanalyses show 94% SHCL-3/B1-positive cells (10,000 cells analyzed, *x*-axis: log green fluorescence, *y*-axis: log red fluorescence, cells positive for one fluorochrome only are on the *x*- and *y*-axis, respectively. Calculation was performed with constant limits for the detection of double-marker–positive cells).

the assay to detect small numbers of HCs. The SHCL-3/B1 test is valuable since HCL can often present with very few symptoms and few circulating HCs. Furthermore, the TRAP test that has been used extensively as a marker of HCL in both clinical and experimental situations is not totally specific for HCs and TRAP-negative HCL cases have been described,[10] and the SHCL-3/B1 assay seems to identify such a TRAP-negative subset of HCs (Fig 5). On the other hand, in three TRAP-positive cases, fewer SHCL-3/B1-positive cells were observed (Fig 5). This can easily be explained, however, by the already mentioned underestimation of double-marker–positive cells. Indeed, microscopic evaluation revealed a much closer correlation to TRAP reactivity in these cases (data not shown).

Attempts to raise Mabs against HCs have yielded antibodies with selective reactivity against either myelomonocytic cells or B cells and no single Mab can yet be claimed to identify HCs.[9,21-24] The value of this double-marker test should be evaluated in light of these findings. Choosing malignant blood diseases known to be positive to either SHCL-3 or B1, we demonstrated that only insignificant numbers of double-marker–positive cells can be found in such situations, and analysis of nonmalignant samples has demonstrated minute but consistent numbers of positive cells (Table 3). Present studies in our laboratory are aimed at the characterization and purification of this small nonmalignant counterpart of the HCs since the data given in Fig 4 have shown that this test is powerful enough to enable sorting of HCs even when they constitute as little as 1% of a cell suspension. The number of SHCL-3/B1-positive cells in our study (mean 0.5%) in 25 normal persons differs only slightly from the number of SIgG+ cells found in peripheral blood by Machii and Kitani (0.9%)[25] and these SHCL-3/B1-positive cells are likely to be the same population as the SIgG+ cells.

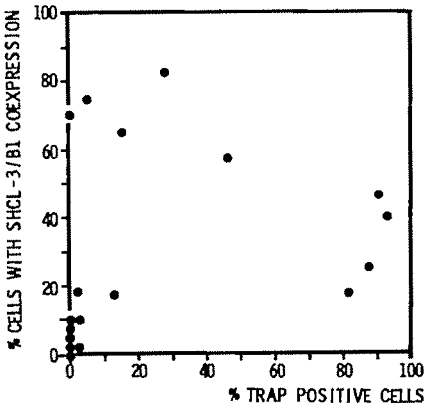

Fig 5. Comparison between immunologic and cytochemical identification of HCs: SHCL-3/B1 reactivity and TRAP positivity were performed on identical MNC suspensions (18 samples from nine patients). The reference criterion for HCs was classical morphology.

Figure 5 clearly indicates that TRAP-negative cases of HCL might be disclosed by use of SHCL-3/B1 analysis, thus

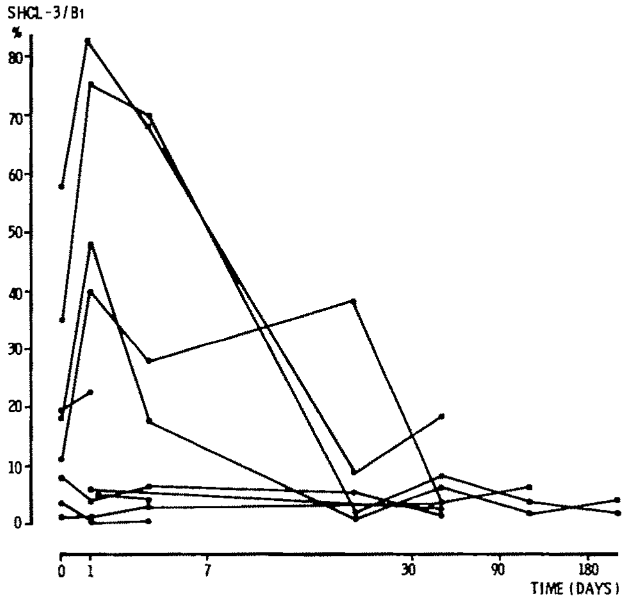

Fig 6. Ten patients receiving recombinant α-IFN (2×10^6 U/m^2 three times a week) were monitored by B1/SHCL-3 analysis.

contributing significantly to the diagnostic procedures in HCL. Based on the longitudinal clinical testing performed in HCL patients receiving IFN (Fig 6) we conclude that this assay is valuable both in diagnosis and monitoring of HCL patients. The assay might be envisaged to be of special value in patients treated with Pentostatin (2′-deoxycoformycin), of whom >50% are believed to enter complete remission.[26] Finally, we emphasize that since the FITC and PE dyes used here are identifiable in the fluorescence microscope, this double-marker assay can also be used in laboratories without access to flow cytometers.

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Establishment of an Interleukin 2-Dependent Human T Cell Line From a Patient With T Cell Chronic Lymphocytic Leukemia Who Is Not Infected With Human T Cell Leukemia/Lymphoma Virus

By Toshiyuki Hori, Takashi Uchiyama, Mitsuru Tsudo, Hiroshi Umadome, Hitoshi Ohno, Shirou Fukuhara, Kenkichi Kita, and Haruto Uchino

We established an interleukin 2 (IL-2)-dependent human T cell line, Kit 225, from a patient with T cell chronic lymphocytic leukemia (T-CLL) with OKT3+, −T4+, −T8− phenotype. Southern blot analysis showed that Kit 225 is not infected with human T cell leukemia/lymphoma virus (HTLV) type I or II, and is probably derived from the major clone in the fresh leukemic cells. Kit 225 cells express a large amount of IL 2 receptors constitutively and their growth is absolutely dependent on IL 2. No other stimuli, such as lectins or antigens, are required for maintaining the responsiveness to IL 2. As abnormal IL 2 receptor expression was also seen originally in the fresh leukemic cells, the establishment of this cell line with IL 2 suggests that IL 2-mediated T cell proliferation is involved in the leukemogenesis of some cases of HTLV-negative T-CLL.

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T CELL CHRONIC lymphocytic leukemia (T-CLL) is a heterogeneous disease clinically, morphologically, and immunologically.[1-5] Although it is believed to be a monoclonal expansion of mature peripheral T cells, the pathophysiology of this disease remains unclear. Our previous study showed that leukemic cells from T-CLL patients with an OKT3+, −T4+, −T8− phenotype express the interleukin 2 (IL 2) receptor without any stimuli and proliferate in response to IL 2.[6] Because the IL 2/IL 2 receptor system plays an essential role in regulation of T cell growth, it is important to know whether failure of this regulatory mechanism is involved in some T cell malignancies. Recent studies[7-9] revealed a close association between the abnormal expression of IL 2 receptor and the infection of human T cell leukemia/lymphoma virus type I (HTLV-I),[10,11] which is believed to be a causative agent of adult T cell leukemia (ATL).[12] The mechanism and role of abnormal expression of IL 2 receptor in HTLV-negative T-CLL has not been well analyzed, however. It appears to be due partly to the lack of appropriate cell lines derived from T-CLL patients. In the present study we established an HTLV-negative and IL 2-dependent T cell line with T4+ phenotype from a T-CLL patient, which was demonstrated to be the same clone as the primary leukemic cells. Using this cell line as a model system, we discuss the implication of IL 2-mediated T cell proliferation in the leukemogenesis of some HTLV-negative T4+ T-CLL cases.

MATERIALS AND METHODS

Patient A 62-year-old man was admitted on September 26, 1983, because of persistent lymphocytosis, systemic lymph node swelling, and erythroderma. Peripheral blood analysis revealed WBC count of 291×10^9/L (leukemic cells 90%), RBC count of 4.81×10^{12}/L, and platelet count of 3.82×10^{11}/L. Bone marrow infiltration of 40% pathologic cells was seen. The serologic test was negative for the anti-ATLA antibody.[12] The diagnosis of T-CLL was made on the basis of clinical features, hematologic characteristics, and cell surface phenotypes.[6]

Cell surface marker analysis. Cell surface antigens were detected by indirect immunofluorescence staining and cytofluorometry as described previously.[6] Monoclonal antibodies OKT3, −T4, −T6, −T8, −T9, −T11 and −Ia were obtained from Ortho Diagnostic Systems (Westwood, MA). Anti-Tac monoclonal antibody (appropriately diluted ascitic fluid) was used as an anti-IL 2 receptor antibody.[13,14]

Cell separation and culture. The patient's peripheral blood mononuclear cells were separated from heparinized blood by Ficoll-Conray density gradient centrifugation Separated leukemic cells were then cultivated in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo) supplemented with 10% fetal calf serum (FCS) (GIBCO, Grand Island, NY), 20 μg/mL tobramycin, and 20% crude IL 2 (culture supernatant of lectin-stimulated human spleen cells) at 37°C under a humid atmosphere with 5% CO_2. After 3 months, crude IL 2 was replaced by 1 U/mL recombinant human IL 2 generously provided by Takeda Chemical Industries Inc. (Osaka, Japan).

Radiolabeled IL 2 binding assay Radiolabeled IL 2 binding assay was done as described elsewhere[15] to determine the number of both high-affinity and low-affinity binding sites

Growth pattern of the cell line cells. To estimate the growth pattern of the cells, 2×10^5 cells in 4 mL culture medium with or without 10 U/mL recombinant human IL 2 were cultured in a set of seven Petri dishes, and the number of viable cells was counted by trypan blue dye exclusion every 24 hours for 7 days.

Southern blot hybridization For detection of HTLV-I or HTLV-II provirus and abnormalities of IL 2 receptor gene and for analysis of clonal identity of the fresh leukemic cells and the established cell line, Southern blot hybridization was performed as described previously.[16] In brief, high-mol-wt DNA was extracted, digested with restriction endonuclease (*Eco*RI), separated by agarose gel electrophoresis, and tranferred onto a nitrocellulose membrane. After being baked, the membrane was hybridized with the nick-translated probes, washed, and autoradiographed As the probes, *Acc*I-*Sma*I fragment of HTLV-I (kindly provided by Dr T.

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Table 1. Cell Surface Markers

Source	Positive Cells (%)							
	OKT3	T4	T6	T8	T9	T11	Ia	Tac
Fresh leukemic cells	91	76	0	5	4	99*	84	4
Kit 225	69	96	0	0	63	97	94	96

*Determined on a freshly thawed sample of frozen cells

Honjo, Kyoto University, Kyoto),[17] LTR of HTLV-II (kindly provided by Dr K. Shimotohno, National Cancer Center, Tokyo), Sau 3A fragment of IL 2 receptor cDNA (Dr T Honjo),[18] and *Hind*III-*Eco*RI fragment of T cell receptor β chain cDNA (Dr T. Honjo)[17] were used.

Chromosomal analysis. Cells in log phase of IL 2-dependent growth were exposed to 0.05 μg/mL Colcemid for 1 hour. These cells were suspended in 0 075 mol/L KCl for 20 minutes at 37°C and then fixed with acetic acid/methanol (1 3). They were spread on slides to air-dry, and the chromosomes were stained with trypsin-Giemsa.

RESULTS

Hematologic and immunologic features of the fresh leukemic cells. May-Giemsa staining revealed large leukemic cells with azurophilic granules in abundant cytoplasm. The cell surface phenotype was OKT3+, −T4+, −T8−, as shown in Table 1. Fresh leukemic cells expressed a small amount of IL 2 receptors recognized by anti-Tac monoclonal antibody, and proliferated in response to IL 2 as was seen in normal phytohemagglutinin (PHA)-stimulated T cells (data shown as patient 2 in Fig 3 in the previous report.[6]).

Long-term culture of the leukemic cells with IL 2. Separated leukemic cells soon began to proliferate in the presence of 20% crude IL 2. After 3 months, crude IL 2 was replaced by 1 U/mL recombinant human IL 2, and the cells continued to grow in an IL 2-dependent manner. The established cell line, named Kit 225, has been cultured for >21 months. As shown in Fig 1, Kit 225 cells proliferated exponentially in the presence of IL 2 but died within several days without IL 2. No other stimuli such as antigens or lectins were required for maintaining the responsiveness to IL 2.

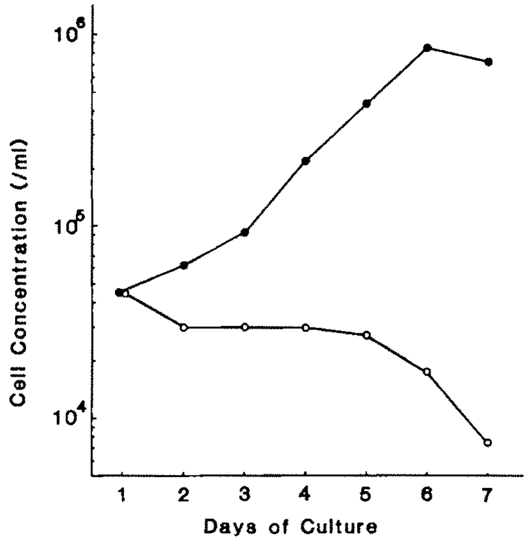

Fig 1. Growth curves of Kit 225 cells. Cells were cultured in medium alone (○) or in the presence of 10 U/mL recombinant human IL 2 (●).

Cell surface phenotype and IL 2 receptor expression. Kit 225 had the cell surface phenotype of OKT3+, −T4+, −T8−, which was essentially the same as the fresh leukemic cells (Table 1). However, intense expression of Tac antigen/IL 2 receptor was noted in Kit 225 as compared with the fresh leukemic cells. To determine the number of IL 2 receptors, radiolabeled IL 2 binding assay was performed and Scatchard analysis revealed that Kit 225 cells expressed 3,000 receptors/cell with high affinity (kd 66 pmol/L) and 190,000 receptors/cell with low affinity (kd 7.0 nmol/L) (Fig 2).

Southern blot analysis. High-mol-wt DNA prepared from the fresh leukemic cells and Kit 225 cells was subjected to Southern blot analysis. As shown in Fig 3, Kit 225 cells have no HTLV-I or HTLV-II proviral DNA integration and have a rearrangement pattern of T cell receptor β chain gene identical to that of the fresh leukemic cells, suggesting that Kit 225 was derived from the major clone in the primary leukemic cells. The latter was supported by the results in another restriction enzyme (*Hind*III) digestion (data not shown). Neither gross rearrangement nor amplification of the IL 2 receptor gene was detected in the fresh leukemic cells and Kit 225 cells.

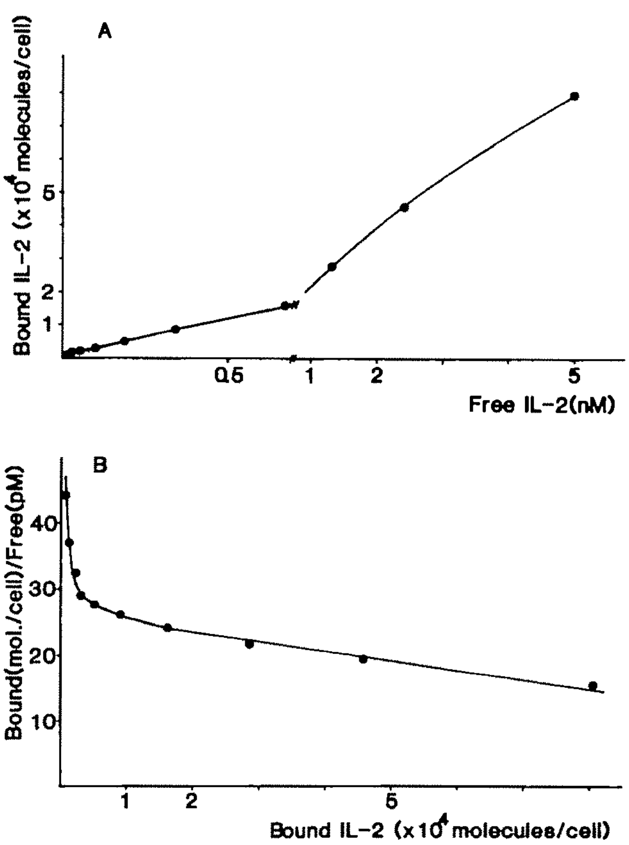

Fig 2. (A) Radiolabeled IL 2 binding to Kit 225 cells. (B) Scatchard plot of the radiolabeled IL 2 binding data shown in A. Estimated numbers of high-affinity and low-affinity IL 2 receptors were 3,000/cell and 190,000/cell, respectively.

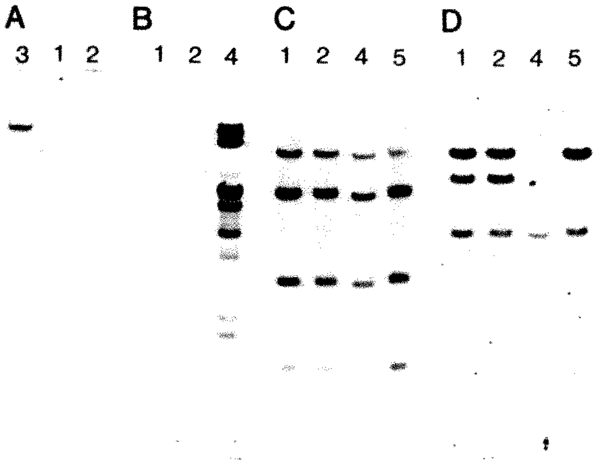

Fig 3. Southern blot analysis of the fresh leukemic cells and Kit 225. High-mol-wt DNA was prepared from the fresh leukemic cells (lane 1), Kit 225 (lane 2), adult T cell leukemia cells (lane 3), Mo, an HTLV-II–infected T cell line (lane 4), and normal human placenta (lane 5). Five micrograms of each DNA sample was digested with restriction enzyme *Echo*RI, separated by agarose gel electrophoresis, and transferred onto a nitrocellulose membrane. After being baked, the membrane was hybridized with the probes of HTLV-I (panel A), HTLV-II (panel B), IL 2 receptor (panel C), and T cell receptor β chain (panel D).

Chromosomal analysis. The karyotype is described in detail (International System for Human Cytogenetic Nomenclature-High Resolution Banding, 1981) as follows: 47, XY, −5, −6, −14, +19, ins inv(1)(pter → p36.3::p34.1 → p31.2::p36.3 → p34.1::p31.2 → p22.1::q12 → p22.1::q12 → qter), inv(3)(pter → p26.2::q27.1 → p26.2::q27.1 → qter), +der(5)t(5;7)(7qter → 7q22::5p15.3 → cen → 5qter), del(6)(pter → q21::q23.3 → qter), +der(6)t(6;?)(q21;?), +der(14)t(6;14)6q21 → 6q23.3::14p12 → cen → 14qter). No abnormalities were detected in chromosome 10, where the gene that encodes IL 2 receptor is located.[19]

DISCUSSION

In the present study, we established an IL 2-dependent human T cell line, Kit 225, from a T-CLL patient with T4+ phenotype. Southern hybridization analysis demonstrated that Kit 225 cells are not infected with HTLV-I or HTLV-II and are probably derived from the major clone in the original leukemic cells.

Some murine IL 2-dependent T cell lines exist.[20,21] Also, in humans, many IL 2-dependent HTLV-infected T cell lines have been established, some of which are reported to become independent of IL 2 during the passage of culture. Establishment of HTLV negative and IL 2-dependent human T cell lines that require no other stimulation has not been reported however, as far as we know.

The IL 2/IL 2 receptor system is considered to regulate normal T cell proliferation.[22] T cells activated by antigens synthesize and release IL 2 as well as express IL 2 receptors on their cell surface membranes, and the binding of IL 2 to its receptors induces the clonal expansion of T cells. The two phenomena, IL 2 production and IL 2 receptor expression, always occur transiently in activated T cells in a normal state, which prevents unlimited growth of T cells. Conversely, it is quite possible that some failure of the IL 2/IL 2 receptor system may cause a neoplastic T cell proliferation.

We previously reported that leukemic cells from most HTLV-negative T-CLL patients with OKT3+, −T4+, −T8− phenotype express IL 2 receptor without any stimuli and proliferate in response to IL 2.[6] Therefore, they seem to be ready to grow by the paracrine mechanism wherever IL 2 is available, even in in vivo environments. In this context, the establishment or immortalization of an IL 2-dependent T cell line, Kit 225, from a T-CLL patient suggests that IL 2-mediated T cell proliferation is involved in the leukemogenesis of some HTLV-negative T-CLL cases with T4+ phenotype. So far, we have succeeded in establishing an IL 2-dependent T cell line from one of eight T-CLL cases we tried. The duration of maintained cell cultures from the remaining seven cases varied from 2 to 16 weeks, which may reflect the heterogeneity of T-CLL in IL 2-dependent growth of leukemic cells.

Recent studies revealed a close association between abnormal expression of the IL 2 receptor and HTLV-I or HTLV-II infection. In such cases, pX region of the virus is speculated to play a key role in augmenting the IL 2 receptor gene expression.[9] However, the mechanism underlying the abnormal expression of the IL 2 receptor on Kit 225 cells as well as HTLV-negative leukemic cells from T-CLL patients remains to be clarified. Kit 225 is expected to serve as a model system of the abnormal expression of IL 2 receptor that may trigger the development of some HTLV-negative T-CLL cases. Further analyses concerning the mechanism of the unregulated IL 2 receptor expression in Kit 225 will be required, including the search for an unknown virus.

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Nonhomogeneous Distribution of Leukemia in the Bone Marrow During Minimal Residual Disease

By Anton C.M. Martens, Frank W. Schultz, and Anton Hagenbeek

In a rat model (BNML) for human acute myelocytic leukemia the distribution of leukemic cells in bone marrow samples from various sites was investigated, using monoclonal antibodies (MoAbs) and flow cytometry. Rats were studied before chemotherapy as well as thereafter, ie, in the "minimal residual disease" (MRD) phase. Bone marrow from different types of bones was analyzed from each animal. Before treatment, the ratio of the measured extreme values (ie, highest/lowest value) for leukemic cell frequencies in bones from individual rats ranged from 3.7 to 11.7. During the MRD phase the ratios of the extremes ranged from a factor of 36 to more than 13,000 from one rat to another. The variability between bones of comparable size was estimated by studying the ribs from each individual animal. Within individuals the extremes differed by a factor of 1.2 to 4.0 before chemotherapy and from 2.4 to >320 after chemotherapy. The variability within the marrow cavity of a single bone was determined by analyzing multiple samples from femoral bones cut into slices. The leukemic cell frequency appeared to vary considerably, ie, before treatment from 1.7 to 7.3 and during MRD from 4 to 28,000. The presented data may contribute to understanding the sometimes conflicting observations in leukemic patients. Improvement of methods for detecting MRD will not automatically lead to a more accurate estimation of the total tumor burden. The reliability of diagnoses based on the analysis of single bone marrow aspirates appears to be highly questionable.

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TREATMENT of leukemia will result in a reduction in the leukemic cell load in the body for most patients. Cell numbers in both bone marrow and blood will drop below the detection level of 5%, and leukemic cells will no longer be distinguishable from normal blast cells by standard cytological criteria. Although the remission-induction success rate for acute leukemia currently reaches 70% to 80%, most patients will relapse within two years,[1] indicating that a substantial number of leukemic cells survive treatment. Early detection of relapse is of utmost importance because the tumor load should be as small as possible at the start of the re-induction treatment.

Methods used to decrease the detection level for leukemic cells include DNA aneuploidy using flow cytometry[2]; cytogenetics[3-5]; immunological methods,[6] and cell culture methods.[7] Immunological methods for the detection of residual leukemia appear to be the most succesful,[8-11] although occasionally the combination of several antibodies[10] and/or lectins[11] is needed to allow for the discrimination of leukemic cells from their normal counterparts.

The lower detection limit for any analytical method is obviously determined by the baseline frequency of cross-reacting normal cells. Using sophisticated, multiparameter flow cytometry, rare cell types such as hematopoietic progenitor cells[12] or residual leukemia cells can be recognized and quantitated up to the level of one cell per 10^4 to 10^5 normal cells.[13-15]

The general assumption that leukemic cells are randomly distributed throughout the bone marrow compartment explains in part why in most leukemia treatment centers only one bone marrow aspirate per patient is investigated during the follow-up period. The result of this single observation is then used to estimate the total tumor burden. Unfortunately, unexpected sudden relapses are a common experience for hematologists.

To get insight into the pattern of distribution of leukemic cells, studies were performed in an experimental rat leukemia model (Brown Norway acute myelocytic leukemia [BNML]). Rats were studied both before and during a cyclophosphamide-induced state of remission. Two assumptions concerning the distribution of leukemic cells within the bone marrow compartment were verified: (1) leukemic cells are randomly distributed throughout the different bones both before and after chemotherapy, and (2) the leukemic cells are randomly distributed within the marrow cavity of single bones.

These observations may help to get an impression of the size, distribution, and population-growth kinetics of the leukemic cell population in the minimal residual disease (MRD) phase. New strategies for detecting residual leukemia in humans may be implied.

MATERIALS AND METHODS

Experimental animals. The experiments were peformed in the barrier-derived inbred Brown Norway (BN) rat strain BNBi/Rij, produced in the Rijswijk colony. Male rats between 13 and 16 weeks of age were used (mean body weight 260 g).

The rat leukemia model. The BNML, induced in a female BN rat by treatment with 9,10-dimethyl 1,2-benzanthracene, shows striking similarites with human acute myelocytic leukemia (AML).[16,17] Upon cellular transfer the leukemia shows a reproducible growth pattern. Some of its major characteristics are: (1) a slow growth rate; (2) severe suppression of normal hematopoiesis due to an absolute decrease in the number of hematopoietic stem cells (colony-forming unit, spleen [CFU-S]); (3) the presence of clonogenic leukemic cells (in vivo leukemic CFU-S [LCFU-S] in vitro clonogenic assays); and (d) response to chemotherapy, as in human AML.

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An additional advantage of this model is that normal hematopoietic stem cells and leukemic clonogenic stem cells can be discriminated by modified spleen-colony assays.

Preparation of cell suspensions. Bone marrow was derived from different bones, ie, femur, tibia, humerus, costa, sternum, vertebra, scapula, and crista up to a total of 16 to 19 specimens per rat. The marrow was flushed with Hanks' Hepes Buffered Balanced Salt Solution (HHBBSS) of 283 mosm, pH 6.8. The larger bones were cut into two parts, each part being flushed several times with HHBBSS. A needle was used to create a small opening in the vertebrae, scapulae, and sternum. The marrow could be collected by flushing these bones with HHBBSS.

Ribs were cut on both sides while care was taken to prevent the loss of bone marrow. The sternal side of the rib was inserted into silicon tubing through which HHBBSS was flushed in order to remove the marrow.

Transverse sections of the femur were made by sawing the bones in several slices using a circular sawing blade mounted on a dental drilling equipment. Cross-contamination of the samples was prevented by cleaning the blade thoroughly in between slices. The marrow was flushed from the bone segment by repeat pipetting of 1 mL of HHBBSS with an Eppendorf-type pipet. Histological examination of bone segments after marrow collection indicated that virtually all cells were removed.

Immunofluorescence labeling of cells. For labeling with the monoclonal antibody (MoAb), cells were centrifuged and resuspended in HHBBSS supplemented with inactivated fetal calf serum (FCS, Flow Laboratories Irvine, Ayrshire, Scotland; 5% vol/vol) and sodium azide (0.01% vol/vol). For fluorescence labeling studies 2×10^6 cells were pelleted and labeled with ascites fluid containing the Fluorescein-iso-thiocyanate (FITC)-conjugated MCA-Rm124 (100 μL) at a $^1/_{243}$ dilution at 0°C for 45 minutes. After 30 minutes of labeling, 25 μL of propidium iodide (PI, Calbiochem, San Diego; 2 μg/μL) were added. PI can easily enter dead cells, in contrast to viable cells, and will subsequently bind to the DNA. This approach enabled the exclusion of dead cells. This is important as dead cells show an aspecific binding of the MoAb, and thus influence the accuracy of the measurements. After careful washing, the cells were resuspended and processed on a modified fluorescence-activated cell sorter (FACS-II).

The Rm124 MoAb. This MoAb was produced by Drs H. Kaizer and R.J. Johnson, Johns Hopkins University, Baltimore. Its affinity for normal and leukemic cells has been discussed elsewhere.[18] Briefly, the MCA-Rm124 reacts with an antigen that is present on leukemic cells as well as on normal granulocytes, although in different densities. Differences in labeling intensity can therefore be used to discriminate between both cell types. Leukemic cell numbers obtained by using the MCA-Rm124 and flow cytometry correlated with data that were obtained with standard bio-assays.[13] This approach proved to be applicable for the detection of minimal residual disease in leukemia, as was reported previously.[13,14]

Flow cytometry. The labeled cells were analyzed with a modified FACS-II (Becton Dickinson, Sunnyvale, CA), with an argon ion laser tuned at 488 nm (0.5 W). Forward light scatter signals (FLS) were used for triggering the measurements. Perpendicular to the laser beam the light was collected on a dichroic mirror DM570 in order to separate "green" FITC fluorescence (<570) and "red" PI fluorescence (>570 nm). The FITC fluorescence was then measured through a broad band multicavity interference filter (520 to 550-nm transmission, Pomfret, Stamford, CT) and a 520-nm cut-off filter (Ditric, Mannheim, FRG) by an S-20 type photomultiplier. A logarithmic amplifier (Designed and constructed by Nozaki, T., Stanford, CA) was used for the FITC fluorescence signals. PI fluorescence was measured through a 620-nm long-pass filter by means of an S-11 type photomultiplier. PI signals were linearly amplified.

On the basis of PI fluoresence the viable cells were identified (negative "red" fluorescence) and "gated out" electronically. The MCA-Rm124/FITC fluorescence of 50,000 to 300,000 cells was measured as described and stored as a histogram in a Hewlett Packard HP87 personal computer (Corvallis, OR).

RESULTS

Leukemic cell frequencies were determined in a great number of bone marrow specimens after labeling with the MCA-Rm124 and measured by flow cytometry as described. The leukemic cells could be specifically recognized and quantified.[13,18] Figure 1 shows typical histograms of a normal bone marrow and of a bone marrow containing a very low number of leukemic cells (ie, one cell per 10,000). The leukemic cells are found in the fraction with the highest fluorescence intensity (indicated by the arrow). To visualize the small leukemic subpopulation, the y-axis is plotted on a logarithmic scale. The leukemic cell frequency can be easily calculated from this type of data by determining the number of cells in the various peaks.

Variability between different rats before treatment. During the development of the leukemia, animals were killed between days 7 and 14 after the intravenous (IV) injection of 10^7 BNML cells. During this period the clinical signs of leukemia are noticeable (increase in weight of spleen and liver, and pronounced infiltration of the bone marrow by the leukemic cells). Marrow cell suspensions of several bones were prepared on day 7 and on day 10 including femora, tibiae, humeri, scapulae, costae, sternum and vertebrae from two animals. The leukemic cell frequencies were determined and ranged from 4×10^{-3} to 5×10^{-2} on day 7 and from 7×10^{-2} to 4×10^{-1} on day 10 after leukemic cell transfer. Obviously, the increase from day 7 to day 10 is a result of

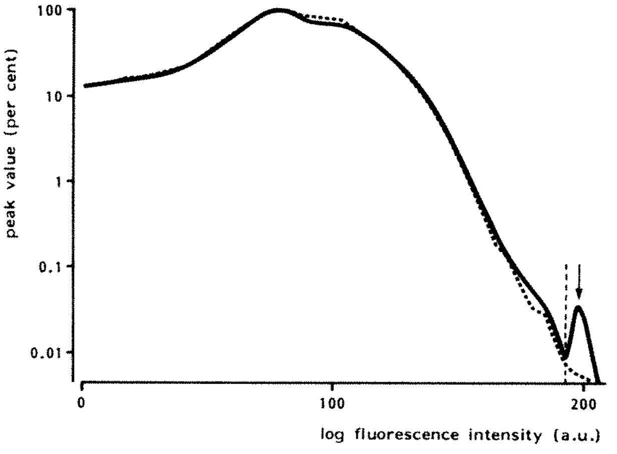

Fig 1. Typical fluorescence intensity distribution of bone marrow from a leukemic rat in remission containing residual leukemic cells (———) compared with normal rat bone marrow cells (– – – –). (Abscissa) Fluorescence intensity (logarithmically amplified); units are arbitrary and relate to channel numbers. (Ordinate) Cell frequency normalized to the percent peak value. The threshold for the "leukemic cell region" is indicated by ----. The arrow points to the residual leukemic subpopulation.

Table 1. Ratios of Measured Maximal and Minimal Leukemic Cell Frequencies in Various Bones of Leukemic Rats Before Cyclophosphamide Chemotherapy

Rat No.	Day After 10^7 BNML	Ratio Highest/Lowest Frequency
1	7	11.7
2	7	6.6
3	10	3.7
4	10	4.4

The bones examined were femur, tibia, humerus, scapula, vertebra, and sternum, up to a total of 16 to 19 specimens.

leukemia growth. The ratio between the highest and lowest measured values of leukemic cell frequencies in the various bones of the individual rats proved to be suitable for comparing animals at different stages of development. Before the administration of chemotherapy the variation of these ratios as determined for all the bones were in a narrow range: 6.6 to 11.7 for day 7 and 3.7 to 4.4 for day 10 (Table 1). The measured leukemic cell frequencies in the various individual bones are shown in Fig 2A.

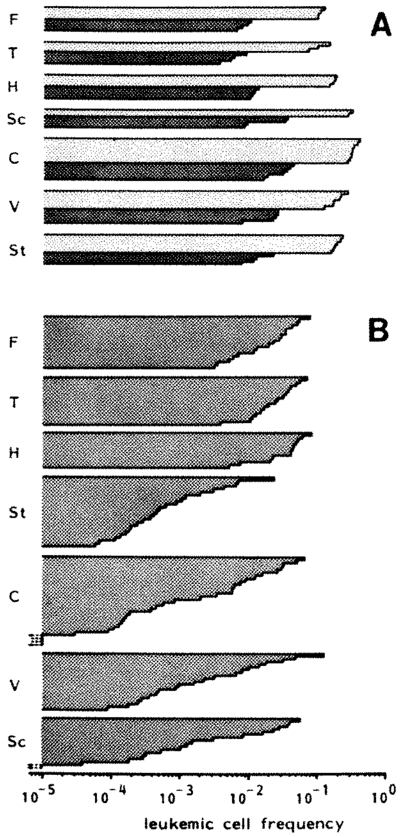

Fig 2. The frequency of leukemic cells in various bone marrow specimens before chemotherapy (A) and during the stage of MRD (B). (A) Pooled data of four rats killed at two different times during undisturbed leukemia growth. Darkly shaded, day 7 after leukemia transfer; lightly shaded, day 11. (B) Pooled data of eight rats killed during the MRD stage, 17 days after treatment (100 mg cyclophosphamide/kg IP) On the ordinate: F, femur, T, tibia, H, humerus, Sc, scapula (of F, T, H, and Sc, two specimens per rat); C, costa, V, vertebra, St, Sternum (of C, V, St, three to four specimens per rat). For each animal a total of 16 to 19 bones were investigated. Small arrows: Samples in which no leukemic cells were detected above the level of 10^{-6}.

Variability between different rats in the MRD situation after treatment. Eight animals were investigated during the MRD phase, which was created as follows: Leukemic animals were treated with a single injection of cylophosphamide (100 mg/kg intraperitoneally [IP]). This resulted in a 5 to 6-log leukemic cell kill.[19] The surviving leukemic cells will regrow and will be detectable again when they are present in numbers higher than one per 10,000 to 100,000 normal cells. Animals were killed starting the seventeenth day after treatment. Large differences between the lowest and the highest ratio values were observed, the lowest ratio being 36, the highest ratio being over 13,000. All other animal ratio values fell between these extremes (Table 2).

The leukemic cell frequencies found for the various individual bones of the eight investigated rats during MRD are shown in Fig 2B. The largest variability was observed in the "smaller bones" such as sternum, costae, vertebrae and scapulae. The highest observed leukemic cell frequency was 1.3×10^{-1}; the lowest observed frequency was below the detection limit of 1×10^{-5}.

Variability in individual rats before treatment. The investigated bones differ considerably regarding their size. As this might have an effect on their leukemic cell content, it was decided to investigate a large number of bones of comparable size from a single rat, ie, the ribs, as it was judged that these would best serve the purpose of studying variability. Therefore, all ribs of individual rats were prepared separately, and the marrow was removed and processed for flow cytometry. Simultaneously, the variability *within* a bone marrow compartment was studied by cutting the femur in sections of about 1 mm in length and subsequently studying the marrow. To study the distribution during undisturbed growth, animals were killed on days 8, 11, or 14 after leukemia-cell transfer. The measured frequencies are shown in Fig 3. In the femur (Figs 3A and B) as

Table 2. Ratios of Measured Maximal and Minimal Leukemic Cell Frequencies in Various Bones During the Phase of Minimal Residual Disease After Cyclophosphamide Chemotherapy

Rat No.	MRD Detection on Day	Ratio Highest/Lowest Frequency
1	29	160
2	30	>3,290* (823)†
3	30	>9,840* (1,100)†
4	30	>13,000* (4,360)†
5	30	>2,500* (281)†
6	31	84
7	31	36
8	31	340
9	31	205

Cyclophosphamide (100 mg/kg IP) was given at day 13 to 14 after 10^7 BNML IV. From each rat 16 to 19 bone specimens were investigated; 50,000 to 300,000 cells per sample were analyzed.

*In case no leukemic cells were found in one or more samples of a series, the calculation of the ratio was performed using the detection limit of 10^{-5} as the lowest frequency.

†The calculated ratio using the lowest frequency of leukemic cells that could be accurately measured in one of the samples of the series.

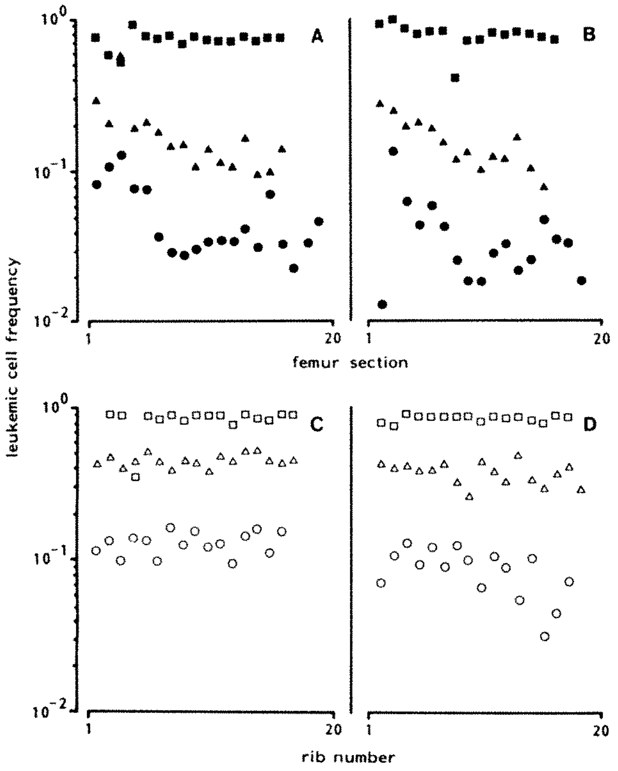

Fig 3. The leukemic cell frequencies in femur and ribs at three different times during undisturbed leukemia growth. Neighboring segments of the femur (A and B) are compared with all ribs of individual rats (C and D) Between 14 and 19 sections were obtained from one femur and analyzed. On the abscissa the femur sections, no. 1 corresponds with the distal end, the highest number with the proximal end of the femur. ■, femur, rat 1 (A) and rat 2 (B) day 14; ▲, femur rat 1 (A) and rat 2 (B) day 11; ●, femur, rat 1 (A) and rat 2 (B) day 7. The ribs are plotted in random order. From each animal 16 to 17 ribs were analyzed. □, ribs, rat 1 (C) and rat 2 (D) day 14; △, ribs rat 1 (C) and rat 2 (D) day 11; ○, ribs, rat 1 (C) and rat 2 (D) day 7.

well as in the ribs (Figs 3C and D) the ratios between the highest and the lowest leukemic-cell frequencies did not vary greatly: from 1.7 to 7.3 for the femur and from 1.2 to 4.0 for the ribs, from one animal to another, considering all three leukemia stages. The ratios of the highest and lowest observed values are given in Table 3. In the femur, during the earlier leukemia stages at days 8 and 11 the ratios were somewhat higher than for the later stage leukemia at day 14: 3.5 to 7.3 *v* 1.7 and 2.4. No specific pattern of distribution across the femoral cavity in the longitudinal direction was observed (Figs 3A and B). However, the average frequency in the femoral sections appeared to be approximately a factor of 2 to 3 lower than for the ribs of the same animal, especially during the earlier stages (compare Figs 3A and B with C and D).

Variability in individual rats in the MRD situation after treatment. Animals were treated with cyclophosphamide on day 8, 11, or 14 after leukemia cell transfer. During the MRD phase leukemic cell frequencies were measured. Large differences were observed between the animals (Table 4).

Table 3. Ratios of Measured Maximal and Minimal Leukemic Cell Frequencies in Femur Sections and in Ribs Before Chemotherapy

Rat No.	Ribs*	Femur Sections*	Day After 10^7 BNML Cells IV
1	2.7	—	7
2	1.3	—	7
3	1.6	5.5	8
4	4.0	7.3	8
5	1.4	—	10
6	1.2	—	10
7	2.8	4.5	11
8	2.9	6.4	11
9	1.6	5.8	11
10	1.9	3.5	11
11	2.7	1.7	14
12	1.2	2.4	14

*See Materials and Methods section for explanation.

The ratios of highest and lowest frequency for the ribs ranged from 2.4 to 70. However, a number of ribs did not contain leukemic cell numbers above the detection limit. Therefore, the calculated ratio will be an underestimation; the maximal ratio which was estimated was even >320.

In the femur sections of the individual animals the ratios ranged from 4 to 1,900. However, in the femur sections, too, in some cases, no leukemic cells were observed, which would imply a possible ratio of >28,300.

DISCUSSION

During the post-chemotherapy period single bone marrow apsirates from leukemic patients are routinely screened in order to verify the state of complete remission or to detect a relapse of the disease as early as possible. As 5% blast cells is considered the borderline for diagnosing leukemia, this leads to difficulties in determining the remission/relapse status of patients being treated for leukemia.[20-23] This implies that the

Table 4. Ratios of Measured Maximal and Minimal Leukemic Cell Frequencies in Femur Sections and in Ribs of Rats During the Phase of Minimal Residual Disease After Chemotherapy (Cyclophosphamide 100 mg/kg IP)

Rat No.	Ribs	Femur Sections	Day of Treatment	Day of MRD Detection
1	ND	18	14	24
2	ND	55	14	24
3	29	4	14	31
1	38	202	11	29
2	>220* (70)†	>28,300* (1,900)†	11	31
3	>84*	‡	11	31
4	35	350	11	38
1	7	7	8	35
2	>320*	66	8	35
3	2.4	>30* (28)†	8	39

Of each sample, 50,000 to 300,000 cells were analyzed.

Abbreviation: ND, not determined.

*In case no leukemic cells were found in one or more samples of a series, calculation of the ratio was performed using the detection limit of 10^{-5} as the lowest frequency.

†The calculated ratio using the lowest frequency of leukemic cells that could be accurately measured in one of the samples of the series.

‡All samples were below the detection limit.

patient may host many leukemic cells, ie, up to 5×10^{10}, not being detected. On the other hand, if leukemic cells are found in the bone marrow aspirate, this is not necessarily representative for other bone marrow sites.

In the rat leukemia model BNML, in undisturbed leukemia growth, the differences in the leukemic cell frequency vary, although not greatly, between different bone marrow sites (Table 1, Fig 2). After chemotherapy, considerably larger differences (in the range of 10,000-fold) in leukemic cell content between various bones were observed (Table 2). The largest variation was observed in the "smaller" type of bones, *eg*, ribs, vertebrae, scapulae, and sternum (Fig 2B). Larger bones, *eg*, the femur, tibia, and humerus, showed a more uniform pattern (Fig 2B). To exclude influences of size, type, and location of the bones on the measured leukemic-cell contents, all ribs of individual rats were investigated (Figs 3C and D). From the same rats the femur, representing the "large-bone" type, was investigated for possible inhomogeneous distribution within the marrow cavity. Small differences were observed (Figs 3A and B, Table 3). When comparing the average leukemic-cell frequency in the marrow of ribs and femur before the animals received chemotherapy, it was striking that especially during the earlier stages (ie, days 7 and 11) in the femur a two- to threefold lower leukemic-cell frequency was observed (Fig 3). This most likely reflects differences in the initial homing after leukemia-cell transfer as the population growth parameters of the leukemic cells in rib and femoral bone marrow were compared and proved to be identical. As a result of chemotherapy, two patterns of response were identified. Animals treated in early-stage (day 8) or late-stage (day 14) leukemia showed a rather uniform pattern of distribution of the leukemic cells at analysis during the MRD stage. They presented with comparable leukemic-cell frequencies at the investigated bone marrow samples and showed low ratio values (Table 4). The largest variation was observed in the "medium stage" leukemia group treated on day 11. To explain this phenomenon the following has to be considered: In "early" stage leukemia relatively few leukemic cells are present in the ribs (in order of 2×10^5). Treatment with cyclophosphamide (100 mg/kg IP) will result in 5 to 6-log cell kill. Consequently, most ribs will be cleared of leukemic cells. Leukemia will be detectable in the ribs again only after migration of cells from elsewhere, which is apparently a random process. In "late stage" leukemia a rib contains about 6×10^6 leukemic cells. Thus, the cyclophosphamide treatment will result in the survival of some cells in most of the ribs, which will subsequently regrow. Therefore, most ribs will have a similar leukemic-cell content at the time of investigation during the MRD phase. In "medium stage" leukemia, however, some ribs will be cleared by the cyclophosphamide treatment while in other ribs cells are surviving that will immediately start to regrow. In the "disease free" ribs, however, cells first have to immigrate, a process with an apparent time delay. This will result in larger differences at the time of investigation compared with the two situations discussed previously. A number of factors are involved such as size of the leukemic cell population, treatment efficacy, size of the bone, distribution within the bone, etc. These will undoubtedly also play a role in the treatment of human leukemia where, not predicted by the outcome of any previous bone marrow examination, totally unexpected relapses occur. That the phenomenon of focal regrowth will be the underlying reason is well recognized.[20-23] To what degree differences in tumor-cell frequency may exist, however, is unknown. The results obtained with this animal model suggest that they can be as high as a factor of 10^5.

Concerning the "ultimate" lower detection limit of leukemic cells in human marrow, the following has to be considered. The lower detection limit can be expected to be in the order of one per 10^5 to 10^6 cells. For statistical reasons 10 to 100 "positive events" have to be scored. This means that 10^7 to 10^8 cells will have to be analyzed. This corresponds with the average number of cells in a good quality punctate, which will have to be totally processed by means of flow cytometry. The entire bone marrow compartment contains about 10^{12} cells. Assuming a random distribution, the leukemic cell load may be a factor of 10^4 to 10^5 times larger than observed in the punctate. If the leukemic cells are nonrandomly distributed the tumor load may be much larger. That this situation may exist is clear from the animal data. If one realizes that frequency differences of up to 10^5 from one site to the other were found, it is clear that relying on single bone marrow aspiration analysis should be discouraged. Multiple bone marrow samples from different sites will increase the chances of detection and quantification of residual disease.

Extended studies in animal models, aiming at a mathematical description of leukemia growth before and after chemotherapy, will be most valuable in formulating a new strategy for optimizing the methods of detecting "minimal residual disease."[24]

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Analyses of Phenotype and Genotype in Acute Lymphoblastic Leukemias at First Presentation and in Relapse

By Anand Raghavachar, Eckhard Thiel, and Claus R. Bartram

As a clue to the cellular origin of leukemic populations in relapse we analyzed 11 cases of acute lymphoblastic leukemia (ALL) by immunological and molecular genetic approaches. Blast cells obtained from both initial diagnosis and relapse were immunophenotyped using a variety of monoclonal antibodies; simultaneously we hybridized Southern blots of respective cell samples to immunoglobulin (Ig) heavy and light chain as well as to T-cell receptor β-chain ($T\beta$) sequences. While similar phenotypes were observed in both states of nine cases, comparison of Ig gene rearrangements revealed clonal variations, ie, appearance of an evoluted or novel leukemic cell clone in relapse beside identical leukemic populations in both states. One pre-T (ALL) patient, presenting with germline configuration of $T\beta$ gene sequences at diagnosis, exhibited a rearrangement of $T\beta$ gene sequences in recurrent disease. Another patient displayed T-ALL phenotype and $T\beta$ gene rearrangement at diagnosis but relapsed with a very immature phenotype and germline configuration for $T\beta$ sequences. Our results emphasize the value of molecular analyses in order to unravel the nature of leukemic relapse.

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THE DIFFERENCES in clinical outcome and the pathogenesis of leukemia relapse are still poorly understood. Several techniques have been used to study the relationship of acute lymphoblastic leukemia (ALL) cells at first presentation and relapse, namely (1) detection of shifts in cell membrane phenotypes,[1] (2) comparative chromosomal analyses,[2] and (3) G-6-PD studies.[3] We investigated 11 ALL patients combining immunological and molecular analyses to determine if genotype and phenotype in malignant cells at relapse were identical to that at diagnosis. Using this approach we could provide compelling evidence that despite a similar phenotype at relapse, leukemic cells may be related but not identical to the original leukemic cell clone.

MATERIALS AND METHODS

Cell samples were obtained from 11 ALL patients who were undergoing routine diagnostic immunophenotyping studies under protocols approved by the Bundesministerium für Forschung und Technologie.[4] These patients were selected because sufficient cryopreserved material for DNA extraction from both initial diagnosis and first relapse was available. Clinical features are summarized in Table 1. Smears of peripheral blood and bone marrow were stained by standard techniques including Wright–Giemsa, periodic acid–Schiff reagent (PAS), myeloperoxidase (MPO), acid phosphatase (AP) and naphtol AS-D chloroacetate esterase (CAE), and characterized according to the French–American—British (FAB) classification.[5] Enriched preparations of blast cells were obtained by Ficoll–Hypaque gradient separation and resulted in $>90\%$ blast cells in all specimens studied. Immunologic phenotypes were determined with monoclonal antibodies B1, B4, J5 (Coulter Immunology, Hialeah, FL), Leu9 (Becton Dickinson, Sunnyvale, CA), OKT3, OKT6, OKT11, OKIa (Ortho Pharmaceutical Corp, Raritan, NJ), BA-1, BA-2 (Hybritech Incorp, San Diego) and VIM-2 (generously provided by Dr W. Knapp, Vienna). Binding of monoclonal antibodies to targets was assessed by indirect immunofluorescence procedures as described.[6] The presence of terminal deoxynucleotidyl transferase (TdT) was examined by indirect immunofluorescence as described by Bollum.[7]

DNA was extracted from cryopreserved leukemic cell samples by standard techniques. Fifteen micrograms DNA was digested with appropriate restriction enzymes (Boehringer Mannheim), electrophoresed on 0.7% agarose gel, blotted and hybridized as described.[8] λ-DNAs were included as molecular weight standards. To demonstrate Ig-gene rearrangements, *Bam*HI digests were hybridized to a 1.3 kb *Eco*RI $C\mu$ probe, which detects a 17-kb germline band[9] (Fig 1A) and a c-k probe, which detects a 12-kb germline band in *Bam*HI digests[9] (Fig 1B). Both probes were kindly provided by Dr P. Leder. *Eco*RI, and *Bam*HI digests were hybridized to a $C_T\beta$ probe detecting 12-kb and 4.2-kb germline bands.[8] (Fig 1C) After hybridization, the filters were washed under stringent conditions and exposed to XAR-5 film (Kodak) using Dupont Lightning-Plus intensifying screens for up to 24 hours at −70°C.

RESULTS

Immunological examination. All patients were classified as having acute lymphoblastic leukemia based on lymphoid morphologic appearance and negative staining for MPO. The results of immunological cell marker studies at the time of diagnosis and at relapse are given in Table 2, following criteria reported elsewhere.[10-12] At first examination, seven patients presented with common ALL, two with pre-T ALL (TdT+, Leu9+, T11−, T6−, T3−), one with T-ALL and one showed acute undifferentiated leukemia (AUL).

Reassessment of leukemia cell phenotypes at relapse revealed that the immunological diagnoses were close to the initial subtype. However, within a given subtype, some minor shifts of the marker pattern in relapse were detected in three patients. The phenotype of pre-T ALL cells in patient no. 10 changed from TdT+ to TdT− and, in addition, the cells became HLA-DR antigen positive. Patient no. 2 (cALL) had a 35% reduction in the proportion of the cells positive for anti-B1 monoclonal antibody at the time of relapse. In patient no. 9, initially diagnosed as having pre-T ALL, an incomplete, more mature T cell phenotype (T6+, T3+, T11−) developed in recurrent disease. Another shift was observed in patient no. 8 who appeared to have a T cell phenotype at presentation and a pre-T phenotype in relapse.

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Table 1. Clinical Features in 11 ALL Patients

Patient No.	Age (yr)/Sex	Time From First Presentation to Relapse (mo)	Immunological Diagnosis at Presentation
1	22/F	5	cALL
2	28/M	24	cALL
3	64/F	3	cALL
4	19/M	14	cALL
5	24/F	24	cALL
6	17/M	4	cALL
7	6/M	16	cALL
8	8/M	6	T-ALL
9	50/M	17	pre-T-ALL
10	29/M	30	pre-T-ALL
11	34/F	5	AUL

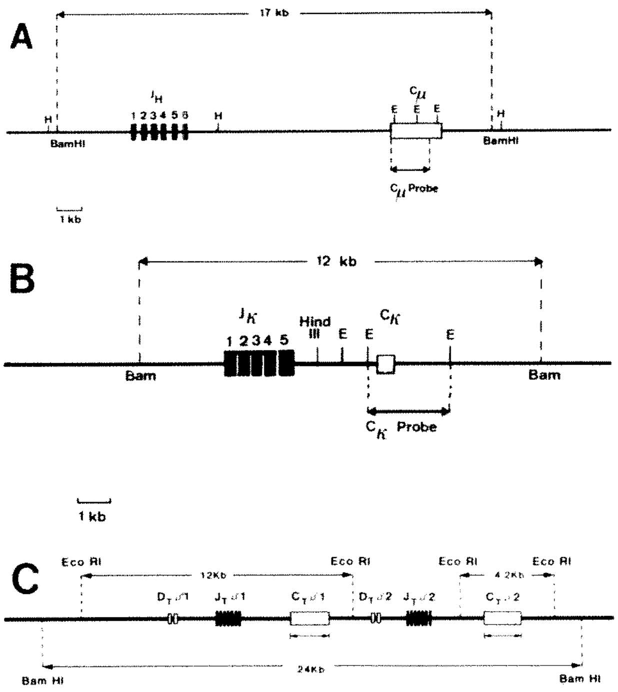

Fig 1. Restriction map of human germline JH-Cμ (A), Ck (B) and TCR (C) loci. Fragments used as probes are indicated in the lower part of the map. H, *Hind*III; E, *Eco*RI.

DNA-analysis. In order to establish the clonal relationships between respective initial and relapsed leukemia we compared Southern blot analyses of both disease states (Fig 2). Of the 11 patients studied, nine showed a rearrangement of Ig–heavy-chain gene sequences (patients no. 1 to 7, 9, and 11). Identical rearranged bands at initial presentation and relapse were detected in four cases (patients no. 1, 3, 4, and 7). Different patterns of Ig-rearrangements in both disease states were detected in five patients. At initial diagnosis, Southern blot analyses revealed a rearrangement of both heavy-chain alleles in patient no. 2. However, in recurrent disease the larger fragment of one allele was only very faintly visible. We interpret this hybridization pattern as an indication for a deletion of Cμ sequences from one allele in the leukemic population in relapse; the faintly hybridizing band suggests the concurrent persistence of the initial leukemic clone at a very low percentage. Additionally, it is remarkable that we observed a reduced proportion of B1-positive cells at relapse. In patients no. 6 and 11, the loss of the germline band in recurrent disease also indicates an additional deletion affecting one allele at this state while the other allele shows the same rearranged band. In contrast, in patients no. 5 and 9, Southern blot analysis revealed Ig rearrangements at relapse that could not be observed in the initial state. Moreover, different kappa–light-chain rearrangements were detected in patient no. 5 at both states (Fig 3). In patient no. 6, kappa-light chains were deleted at both instances. Faintly hybridizing bands of germline size in this case represent, in all likelihood, contamination of cell samples with no leukemic cells. In all other patients studied, kappa–light-chain sequences were in germline configuration (data not shown). In patient no. 10, exhibiting germline Ig–heavy-chain sequences at both disease states, examination of Tβ genes was informative; this pre-T ALL lacked Tβ-gene rearrangements initially; however, at relapse, a Tβ gene rearrangement was visible (Fig 4). As mentioned above, blast cells of this state had become TdT-negative, retained a Leu9+ phenotype, but lacked more mature T cell markers. Thus, there was a discrepancy between the genotype and cell surface marker expression. On the other hand, in patient no. 8 in accordance with phenotypic conversion from T- to pre-T-ALL, the genotype changed from rearranged to germline configuration of Tβ-chain sequences. These results are in concordance with the respective phenotypes of the leukemic cells, with the remarkable exception of case no. 9 presenting a T cell leukemia by immunological criteria but displaying rearrangements of both Tβ and Ig sequences. Although this patient exhibited a more mature T cell phenotype at relapse, Southern blot analyses showed identical

Table 2. Immunological Data of 11 ALL Patients Studied During Presentation (a) and in Relapse (b)*

	1		2		3		4		5		6		7		8		9		10		11	
	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b
TdT	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+
BA-1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−
BA-2	−	−	+	+	+	+	−	−	+	+	+	+	−	−	−	−	−	−	−	−	+	+
B4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−
B1	−	−	+	−†	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−
J5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−
OKIa	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	+	+	+
Leu9	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	−	−
OKT3	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
OKT6	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+	−	−	−	−
OKT11	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
VIM-2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+

*+ = >20% staining in indirect immunofluorescence assays.

†B1 positive cells were 38% in (a) and 5% in (b).

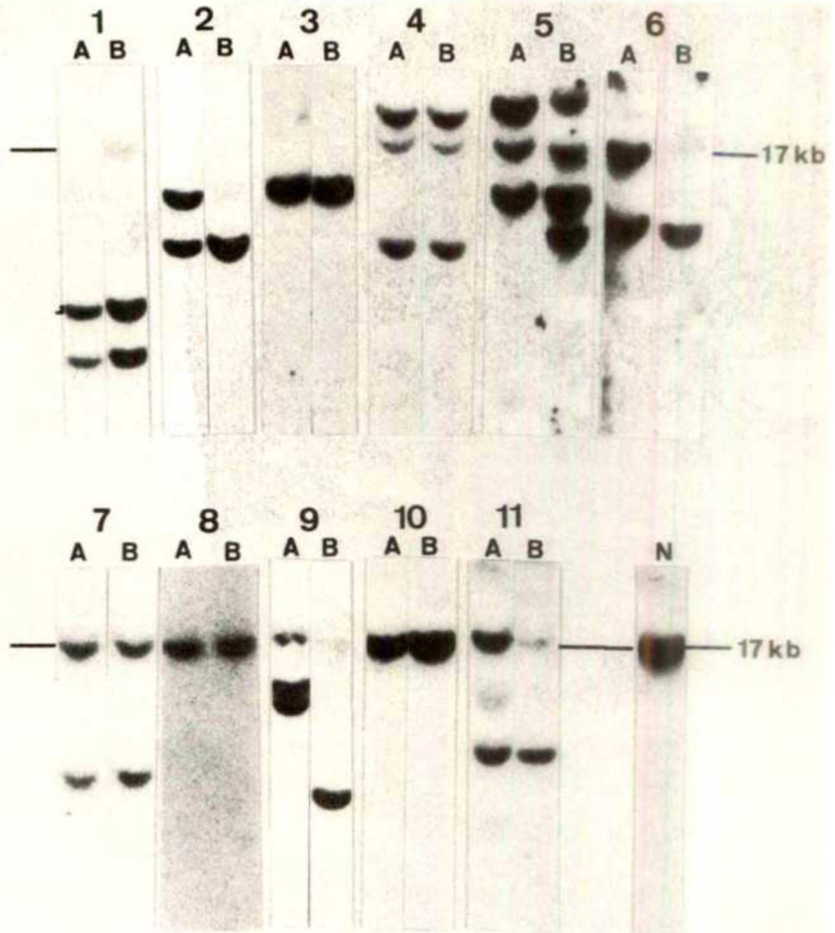

Fig 2. Southern blot analyses of DNAs (15 μg) obtained from leukemic cells of 11 ALL patients at first presentation (A) and relapse (B). *Bam*HI digests were electrophoresed, blotted, and hybridized to a $C\mu$-probe that detects a 17-kb germline autoradiographic band. N = placenta-control DNA.

$T\beta$ rearrangements in both disease states (Fig 4), while the pattern of Ig rearrangements was entirely different (Fig 2) as outlined above. With these three exceptions, in the remaining patients T-β genes were in germline configuration at both instances (not shown).

DISCUSSION

We have reassessed tumor cell pheno- and genotypes in 11 ALL patients in order to establish the clonal relationships between initial and relapsed disease. These patients included different immunological ALL subtypes. Relapses occurred within 3 and 30 months of diagnosis. Our study demonstrates that the immunological subtype in the majority of ALL patients remained virtually identical at initial diagnosis and

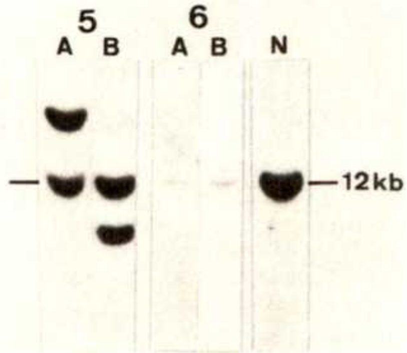

Fig 3. Configuration of kappa-light-chain sequences in patients no. 5 and 6. *Bam*HI digests were electrophoresed, blotted, and hybridized to a c-k probe that detects a 12-kb germline fragment.

Fig 4. Configuration of T cell receptor β-chain sequences in patients no. 8, 9, and 10 at first presentation (A) and relapse (B). *Eco*RI digests were electrophoresed, blotted, and hybridized to $C_T\beta$ sequences that detect 12-kb and 4.2-kb germline bands. N = placenta-control DNA.

relapse. This is supported by previous studies based on more patients.[1,11] However, in former studies, examination of Ig and Tβ-gene rearrangements as a convenient method for determining the ultimate clonality of lymphoid neoplasms have not been performed. Indeed, using this approach we detected marked clonal heterogeneity of leukemic cell clones at diagnosis and relapse. Seven of eleven patients exhibited Southern blot analyses discordant with those found on initial tumors, irrespective of whether these patients had early or late relapses. The possibility that our findings may reflect the evolution of subclones within a single malignant-cell population rather than the occurrence of two independent clones is suggested by the detection of at least one identical rearranged allele in cases no. 2, 5, 6, and 11. However, the discovery of different kappa-gene rearrangements in patient no. 5 makes it difficult to establish that leukemic cells at initial diagnosis and relapse are clonally related. We cannot rule out the possibility that in the relapse of this patient a novel leukemic cell clone emerged characterized by distinct cμ and c-k rearrangements, while the original leukemic population persisted but molecularly evolved, visible by monoallelic deletions affecting cμ and c-k sequences. In this respect, recent reports might be of interest, providing strong evidence for the presence of more than one leukemic cell clone in ALL at first presentation[10,13] or relapse.[14] The clonal relationship in patients no. 8 and 10 cannot be sufficiently settled by our molecular genetic studies since these patients shared no identical gene rearrangements at both instances. In principal, cytogenetic analyses might be of help in such instances but were unavailable for our cases.

In a recent report cytogenetic analyses of changes from diagnosis to relapse revealed the presence of independent cell clones in two of 51 ALL patients.[2] In this context, our data might provide support for the view that leukemic cells of some cases in relapse may be related but not identical to the original leukemic clone. Studies of G-6-PD phenotypes in Burkitt's lymphoma by Fialkow et al[16] further support this notion. In this context, the dual rearrangements in patient no. 9 might be of particular interest. The pre-T-ALL phenotype of this patient at diagnosis is in keeping with the recent notion that patients with precursor T cell leukemia show a greater tendency to rearrange both Ig and TCR genes.[15,17] The explanation for this cross-lineage rearrangement may be due to the use of a common recombinase in B and T cells.[18] The identical rearranged TCR sequences (but completely different Ig rearrangements at relapse) show the clonal relationship of both leukemic populations but raise some interesting questions concerning the regulation of Ig and TCR genes during the early stages of lymphocyte differentiation. In such situations recombinases responsible for Ig and TCR-gene rearrangements may remain active until mature Ig or TCR molecules are produced. In this respect, the molecular mechanism for the Ig-gene variation detected at relapse may include normal developmental progression.

In summary, recognition of changes in Ig- and Tβ-gene rearrangements at the time of leukemic relapse may increase our understanding of the pathogenesis of tumor recurrence and of the selective effects of therapy on neoplastic cell populations. This study represents a first step toward this goal. Investigation and follow-up of more patients (including sequence analyses of respective rearranged TCR and Ig genes) may, therefore, finally reveal the clinical importance of the molecular differences, ie, clonal evolution of independent clones among these patients.

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Asialo-von Willebrand Factor Inhibits Platelet Adherence to Human Arterial Subendothelium: Discrepancy Between Ristocetin Cofactor Activity and Primary Hemostatic Function

By Jeffry B. Lawrence and Harvey R. Gralnick

Platelet adherence at high wall shear rates requires plasma von Willebrand factor (vWF). Clinically, the ristocetin cofactor (RCof) activity is the only widely available assay for vWF function. When purified vWF is treated with neuraminidase to yield asialo-vWF (AS-vWF), its RCof activity is increased by 20% to 40%. AS-vWF binds to normal human platelets independently of ristocetin and induces platelet aggregation in the presence of fibrinogen. To determine whether AS-vWF also shows an enhanced capacity to support platelet adherence to subendothelium, we used the Baumgartner technique. Intact vWF, AS-vWF, or AS-vWF treated with β-galactosidase (asialo, agalacto-vWF; AS,AG-vWF) was added to normal citrated whole blood before perfusion over human umbilical artery segments (wall shear rate, 2,600 sec^{-1}). Four micrograms per milliliter AS-vWF caused a 69% reduction in total platelet adherence compared with citrated whole blood ($P < .001$), and 4 µg/mL AS,AG-vWF led to a 48% reduction ($P < .005$). With 4 µg/mL intact vWF, the platelet adherence values were not significantly different from the controls. No significant differences in subendothelial platelet thrombi or postperfusion platelet counts were evident among any of the groups. In reconstituted afibrinogenemic perfusates, 4 µg/mL AS-vWF caused a 42% reduction in platelet adherence ($P < .05$). Thus, AS-vWF is a potent inhibitor of platelet adherence, despite its enhanced RCof specific activity. Abnormalities in vWF carbohydrate may play a role in impaired primary hemostasis in some patients with von Willebrand's disease.

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PLASMA von Willebrand factor (vWF) is an essential cofactor for platelet adherence to vascular subendothelium at high wall shear rates.[1,2] The molecule must first bind to the subendothelium[1] in order to enable platelets to attach and spread along the subendothelial surface and subsequently form aggregates. The ability of vWF to support the agglutination of platelets in the presence of ristocetin forms the basis for the only widely used assay for the functional activity of plasma vWF. In general, the ristocetin cofactor (RCof) activity has been clinically useful for the evaluation of patients with von Willebrand's disease.[3,4] However, discrepancies between RCof activity and the bleeding time and severity of clinical bleeding have been noted.[5-10] This suggests that the vWF-dependent mechanisms that physiologically mediate platelet-subendothelial interaction in primary hemostasis are not completely reflected in the RCof assay. Since structure-function investigations of vWF have largely relied on the RCof activity,[11-13] extension of the conclusions of these studies to the physiology of primary hemostasis may be somewhat problematic.

vWF is a multimeric glycoprotein whose carbohydrate content comprises 14% of its molecular weight.[14] Approximately 4% of the glycoprotein is comprised of sialic acid residues.[14] When purified vWF is treated with neuraminidase to remove its sialic acid, the resulting asialo-vWF (AS-vWF) demonstrates a 20% to 40% increase in RCof activity.[15] Initial studies by De Marco and Shapiro[16] and subsequent work from our laboratory and others[15,17] have shown that AS-vWF binds to normal human platelets independently of ristocetin and induces platelet aggregation dependent on platelet glycoproteins Ib(GPIb) and IIb/IIIa (GPIIb/IIIa) in the presence of plasma fibrinogen. It has been postulated that small amounts of AS-vWF that may be generated at sites of vascular disruption might enhance platelet adherence to subendothelium and subsequent platelet aggregation in primary hemostasis.[16] To test this hypothesis, we have employed the Baumgartner technique, an ex vivo system that appears to simulate the in vivo events of primary hemostasis,[18] to define whether AS-vWF was associated with enhanced platelet adherence to human arterial subendothelium. We have also treated AS-vWF with β-galactosidase to remove its terminal and penultimate galactose moieties. We compared asialo, agalacto-vWF (AS,AG-vWF) with AS-vWF and the native protein in attempt to address the role of carbohydrate moieties of vWF in its capacity to support platelet adherence.

MATERIALS AND METHODS

Perfusion procedure and morphologic evaluation. Human umbilical arteries were dissected free from umbilical cords obtained immediately after birth. Arterial segments, approximately 15 to 20 mm in length, were everted and de-endothelialized by brief exposure to air. As previously described,[19] the vessels were treated with 0.1 mmol/L aspirin for one hour before being used in experiments, and were then washed four times in 0.2 mol/L Tris-HCl buffer (pH 7.35) to completely remove the aspirin before exposure of the vessels to perfused blood. The vessel segments were pretreated with aspirin to prevent prostacyclin production by vascular smooth muscle cells.[20] An annular perfusion chamber similar to those developed by Baumgartner[18] was used. This device contains a rod on which everted umbilical artery segments are mounted such that the blood entering the chamber flows through the annular space between the subendothelial surface and the chamber wall, thereby exposing the subendothelium to platelets. The chamber has an effective annular width of 0.18 cm. This is the distance between the chamber wall and the central rod, minus the average thickness of the artery wall (~0.15 mm.) Steady, nonpulsatile flow across the chamber was produced by gravity using a plastic funnel connected to a peristaltic pump

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(Varistaltic A Series; Manostat Corp, New York) as previously described.[19,21] For all experiments citrated whole blood (final whole blood concentration 10.88 mmol/L sodium citrate), or reconstituted citrated blood composed of washed platelets, platelet-poor plasma (PPP) and washed red cells, was warmed for five minutes at 37°C and then perfused across the chamber at 37°C and recirculated at a rate of 300 mL/min for five minutes. The corresponding wall shear rate, 2,600 sec^{-1}, closely approximates conditions physiologically present in the microvasculature.[22]

After a perfusion experiment, the arterial segment was washed by perfusing 0.2 mol/L Tris-HCl buffer (pH 7.35) through the chamber for five minutes. The segment was fixed in 4% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4) and cross sections were made from the central 5 to 8 mm portion of the segment. As previously described,[19] the sections were dehydrated in graded ethanol solutions and propylene oxide, embedded in an oriented manner in epoxy resin (LX-112; Ladd Research Industries, Burlington, VT), and stained with toluidine blue and basic fuchsin. 0.8 μm sections were evaluated morphometrically by light microscopy according to Baumgartner and Muggli [18] For each perfusion experiment, two cross sections of the arterial segment were evaluated over their entire surfaces at 10 μm intervals, and the platelet-subendothelial interactions were categorized as either C, contact platelets attached but not spread on the surface, or S, platelets spread on the surface. The latter category may include spread platelets with superimposed platelet aggregates (thrombi) that extend 5 μm or more above the subendothelial surface. Platelet adherence is defined as C + S, and each parameter is expressed as a percentage of the total number of points counted (~1,500) per vessel segment. All values are expressed as the mean ± SEM, and Student's *t* test was used to evaluate the significance of differences between mean values.

Perfusate preparation. Donors for all of the perfusion studies were normal hospital personnel who had no bleeding history and were not receiving medications that interfered with platelet function Pre- and postperfusion complete blood counts were performed on all blood samples by an electronic counting device (Model S Plus; Coulter Electronics, Hialeah, FL). The mean preperfusion values (and ranges) were as follows: hematocrit, 43.6% (39.0% to 46.0%); and platelet count, 329,000/μL (252,000 to 405,000/μl). A portion of the citrated blood drawn from each donor was used in its native state for control perfusions, while purified vWF, AS-vWF, and AS,AG-vWF were added to other aliquots of the sample. These proteins were added to the whole blood immediately before the samples were warmed for five minutes at 37°C before perfusion

For experiments using reconstituted blood, washed platelets and red cells were obtained from a normal donor. and PPP was obtained from a patient with congenital afibrinogenemia. This PPP contained less than 5 μg/mL fibrinogen, as measured by the tanned red cell hemagglutination assay, and had a thrombin time >300 seconds. The washed platelets and sedimented red cells were prepared from whole blood collected in 12% citrate/phosphate/dextrose/adenine anticoagulant (final whole blood concentrations: 1 9 mmol/L citric acid, 11.0 mmol/L sodium citrate, 2.0 mmol/L dextrose, 0.2 mmol/L adenine, 2.0 mmol/L monobasic sodium phosphate) Platelet-rich plasma (PRP) was obtained by centrifugation at 25°C for ten minutes at 200 × g. The PRP was diluted 1:1 in Ringer's citrate dextrose buffer (RCD), pH 6.5, containing 43 ng/mL prostaglandin E_1 (PGE_1; Sigma Chemical Co, St Louis). RCD was comprised of 71.9 mmol/L NaCl, 0.7 mmol/L KCl, 0.6 mmol/L $CaCl_2$, 0.8 mmol/L $NaHCO_3$, 20.0 mmol/L trisodium citrate, 27.8 mmol/L dextrose. The PRP diluted in RCD with PGE was centrifuged at 25°C for 20 minutes at 1,100 × g. The platelet pellet was washed with RCD, pH 6.5, containing 43 ng/mL PGE_1, centrifuged at 25°C for 20 minutes at 1,100 × g, and then washed with RCD, pH 6.5, without PGE_1. After centrifugation at 25°C for 20 minutes at 1,100 × g, the final platelet pellet was resuspended in RCD, pH 7 2, without PGE_1. The sedimented red cells were washed twice in saline dextrose buffer (154 mmol/L NaCl, 111 mmol/L dextrose). The platelets, red cells, and afibrinogenemic PPP were combined to yield a hematocrit of 40% and a platelet count of 246,000 per microliter. Four micrograms per milliliter of purified vWF was added to a portion of this reconstituted blood, and 4 μg/mL of AS-vWF was added to the remainder before perfusion.

Protein purification and carbohydrate modification. The vWF protein was purified from human cryoprecipitate by chromatography on Sepharose 4B as previously described.[23] Protein concentrations were estimated by the method of Lowry et al [24] The sialic acid was removed from intact vWF by the addition of *Clostridium perfringens* neuraminidase (Sigma Chemical Co) as previously described.[15] Briefly, 0.02 to 0.04 U neuraminidase per milligram protein (1 U of enzyme activity defined as the amount that released 1 μmol of sialic acid per minute at 37°C from α_1 glycoprotein) was incubated at 37°C with purified vWF for two hours. The enzyme contained no protease activity when reacted with [^{14}C] globin.[15] Sialic acid was measured by the method of Warren,[25] using N-acetyl neuraminic acid as the standard. The sialic acid released by neuraminidase was quantified by the same method, except that the acid hydrolysis step was omitted.[25] The mean sialic acid content of the vWF protein was 144 ± 15 nmol/mg ($n = 3$). Neuraminidase treatment for two hours released >97% of the sialic acid. The neuraminidase was not removed from the AS-vWF preparations before their addition to the perfusates Therefore, control experiments were performed in which neuraminidase was added to whole blood at a concentration of 004 U/mL, a final concentration 2.5 to 10 times higher than the concentration of neuraminidase present in the AS-vWF perfusates AS-vWF was treated with β-galactosidase, prepared from *Streptococcus pneumoniae* (gift of Dr G. Ashwell, Bethesda, MD), to remove its ultimate and penultimate galactose residues as previously described [26] Briefly, 0.005 U β-galactosidase per milligram protein was added to AS-vWF and incubated at 37°C for eight hours. This enzyme was also protease free.[26] The released free galactose was measured in an assay using galactose dehydrogenase as described,[26] and the total galactose content of vWF was determined by hydrolysis in 2 mol/L hydrochloric acid at 100°C for 90 minutes, followed by assay for free galactose.[26] The mean total galactose measured by acid hydrolysis was 139 ± 24 nmol/mg ($n = 3$). Treatment with β-galactosidase removed approximately 72% of this galactose (range 63% to 79%).

RESULTS

When citrated whole blood from normal donors was perfused over umbilical artery segments for five minutes at a wall shear rate of 2,600 sec^{-1}, morphometric analysis showed approximately 50% of the subendothelial surface to be covered with adherent platelets (Table 1; Fig 1). The majority of this surface coverage was represented by a monolayer of spread platelets (Table 1). Approximately 1% of the surface was occupied by platelet thrombi extending 5 μm or more above the subendothelium.

Addition of 4 μg/mL purified vWF to whole blood from these donors led to platelet adherence values not significantly different from the controls (Table 1; Fig 1). However, addition of 4 μg/mL AS-vWF caused a 69% reduction in platelet adherence ($P < .001$), (Table 1; Fig 1). With 4 μg/mL AS-vWF, platelet spreading (S) was significantly reduced relative to both the controls ($P < .001$) and the perfusates containing 4 μg/mL purified vWF ($P < .005$). By contrast, the AS-vWF significantly inhibited surface cover-

Table 1. Effect of Carbohydrate Modification of Plasma vWF on Platelet Adherence to Human Arterial Subendothelium

	Percentage of Contact (C)	Percentage of Spread (S)	Total Platelet Adherence (C + S)
Controls (n = 10)	1.79 ± 0.20	50.94 ± 4.93	52.73 ± 4.89
4 μg/mL vWF (n = 3)	2.23 ± 0.28	36.71 ± 5.58	38.95 ± 5.83
4 μg/mL AS-vWF (n = 7)	1.25 ± 0.17	14.99 ± 2 09*	16 24 ± 2.12*
4 μg/mL AS, AG-vWF (n = 7)	1.40 ± 0.22	25.75 ± 3.02†	27.16 ± 3.15†

De-endothelialized human umbilical arteries were perfused with citrated blood at 37 °C for five minutes at a wall shear rate of 2,600 sec^{-1} Control samples contained no added plasma proteins Mean values (± SEM) are shown. The indicated values are significantly different from control values.

*$P < .001$.

†$P < .005$.

age with contact platelets (C) relative to perfusates containing purified vWF ($P < .02$), whereas the inhibition compared with controls failed to reach statistical significance ($P > .20$). There was no significant difference in surface coverage with platelet thrombi between any of the experimental or control groups. Platelet adherence was significantly inhibited by doses of AS-vWF as low as 1 μg/mL (Fig 1). When doses of AS-vWF > 4μg/mL were added to whole blood, further reductions in platelet adherence were produced, but at these doses the postperfusion reduction of the platelet count of the perfusate became significantly greater than that observed with control samples. By contrast, at doses up to 4 μg/mL, the postperfusion platelet counts in the AS-vWF experiments were not significantly different from those of the controls (Table 2). Since the neuraminidase was not removed from the AS-vWF preparations before their addition to whole blood, control experiments were performed in which neuraminidase was added to whole blood at a dose of .004 U/mL, a dose 2.5 to 10 times higher than the final whole blood concentration present in the AS-vWF perfusates, immediately before warming the samples for five minutes at 37°C before perfusion. The mean total platelet adherence (C + S) for these experiments (n = 2), 44.58%, was not significantly different from that of the controls (Table 1).

When 4 μg/mL AS,AG-vWF was added to normal whole blood, platelet adherence was reduced by 48%, compared with controls ($P < .005$; Table 1; Fig 1). As with AS-vWF, S was significantly reduced by AS,AG-vWF, whereas the inhibition of C failed to achieve statistical significance (Table 1). AS,AG-vWF caused no significant differences in platelet thrombus formation or postperfusion platelet counts (Table 2) compared with controls.

Since platelet aggregation caused by AS-vWF requires plasma fibrinogen,[15,17] a reconstituted afibrinogenemic perfusate was prepared to define whether the inhibition of platelet adherence observed with AS-vWF resulted from platelet microaggregate formation in the circulating blood. Four micrograms per milliliter of purified vWF was added to a portion of this perfusate and 4 μg/mL of AS-vWF was added to the remainder before perfusion. Even in the absence of plasma fibrinogen, AS-vWF caused a 42% reduction in platelet adherence compared with native vWF ($P < .05$; Table 3). Postperfusion platelet counts in the two groups were not significantly different (Table 3).

DISCUSSION

The observation that low concentrations of AS-vWF are capable of directly binding to and aggregating platelets in the absence of ristocetin led earlier investigators to propose the hypothesis that sialidases or sialyltransferases derived from platelets or other cells may physiologically generate AS-vWF from plasma vWF at sites of vascular injury.[16] The AS-vWF would be deposited at sites of endothelial cell injury and would enhance platelet adherence to the subendothelial

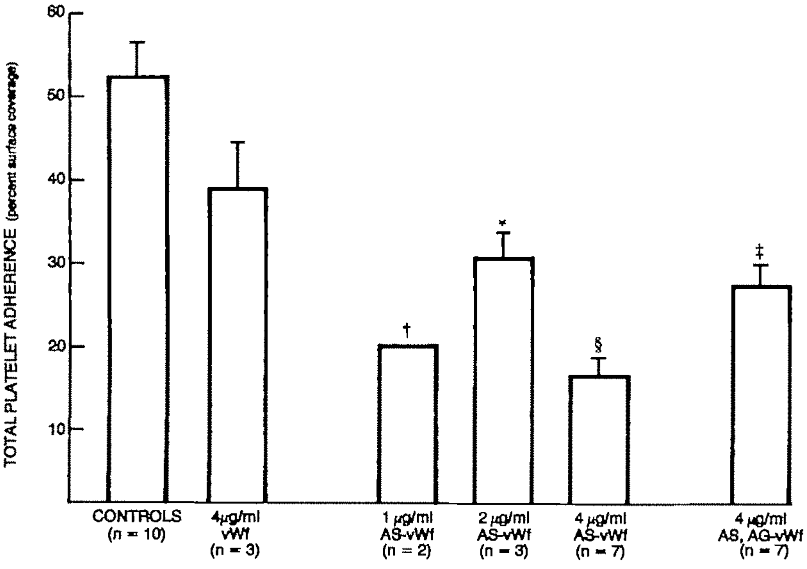

Fig 1. Citrated whole blood samples were perfused for five minutes at 37°C over human umbilical artery segments at a wall shear rate of 2,600 sec^{-1}. These perfusates contained either no added plasma proteins (controls) or the specified amounts of purified vWF, AS-vWF, or AS,AG-vWF. Mean values ±SE are expressed, and statistically significant differences between platelet adherence values are designated: *, $P < .05$; †, $P < .02$; ‡, $P < .005$; δ, $P < .001$.

Table 2. Postperfusion Percentage Reduction in the Platelet Counts of Perfusates Containing Added vWF or Carbohydrate-Modified vWF

Added Protein	Percent Postperfusion Platelet Reduction
Controls (n = 10)	36 ± 3
4 μg/mL vWF (n = 3)	34 ± 2
1 μg/mL AS-vWF (n = 2)	38
2 μg/mL AS-vWF (n = 3)	46 ± 9
4 μg/mL AS-vWF (n = 7)	43 ± 5
4 μg/mL AS,AG-vWF (n = 7)	43 ± 3

De-endothelialized human umbilical arteries were perfused with citrated blood at 37 °C for five minutes at a wall shear rate 2,600 sec^{-1}. Control samples contained no added plasma proteins. Mean values (± SEM) are shown. None of the experimental values showed a statistically significant difference from the controls.

surface at the high wall shear rates in the microcirculation, and subsequent platelet aggregation. This hypothesis is difficult to test in most systems, due to the complexity of events that occur in primary hemostasis. However, the Baumgartner technique permits one to directly examine the role of AS-vWF in platelet adherence. The hypothesis would predict that AS-vWF, when added to normal blood, would cause enhanced platelet adherence. Our data clearly demonstrate a potent inhibitory effect of AS-vWF on platelet adherence (Tables 1 and 3; Fig 1), suggesting that AS-vWF is not an effective physiologic mediator of platelet-subendothelium and platelet-platelet interactions in primary hemostasis.

The mechanism of the inhibition of platelet adherence caused by AS-vWF is not understood at present. Sakariassen et al demonstrated that plasma vWF must first bind to the subendothelium in order to support platelet adherence at high wall shear rates.[1] It is possible that AS-vWF may not bind to the subendothelial surface as readily as native plasma vWF. If this is the case, binding of the AS-vWF to the platelet GPIb and GPIIb/IIIa, as demonstrated by us and others,[15,17] could block these platelet receptors from interacting with the subendothelial-bound native plasma vWF. This would inhibit platelet adherence because vWF binding to GPIb is required for platelets to attach to the subendothelium normally.[19,27-29] Furthermore, we and others have also shown that normal platelet spreading along the subendothelial surface requires intact GPIIb/IIIa.[19,28,29] This mechanism is consistent with our observation that AS,AG-vWF is less potent as an inhibitor of platelet adherence than is AS-vWF (Table 1; Fig 1), for it has been shown that AS,AG-vWF binds with a lower affinity to platelet GPIb than does AS-vWF.[30,31] Thus, AS,AG-vWF would be expected to compete less readily for binding sites on GPIb, and probably the GPIIb/IIIa complex, with subendothelial-bound native plasma vWF, leading to decreased inhibition of normal platelet adherence compared with AS-vWF.

An alternate mechanism to explain the decreased platelet adherence would involve the binding of AS-vWF and AS,AG-vWF to the subendothelium with similar affinities to that of native plasma vWF, yet they may be less efficient in supporting platelet attachment and spreading than the native molecule. Recent studies by Girma et al suggest that the amino-terminal portion of vWF contains distinct sites responsible for binding to GPIb and collagen, while the GPIIb/IIIa binding site appears to be near the carboxy-terminal end of vWF.[32] Thus it is possible that the collagen-binding site on vWF may not be affected by removal of carbohydrate, whereas the GPIb and/or GPIIb/IIIa attachment sites may require a full complement of sialic acid and galactose to maintain the capacity of vWF to support platelet adherence. The observed inhibition of platelet adherence does not appear to be due merely to platelet aggregation causing an effective thrombocytopenia in the perfused blood, since the postperfusion platelet counts in the control and experimental groups were not significantly different (Table 2). Furthermore, in the experiments using afibrinogenemic reconstituted blood, platelet adherence was significantly inhibited by AS-vWF (Table 3), despite the fact that AS-vWF-induced platelet aggregation is dependent on the presence of plasma fibrinogen.[15,17]

Previous studies from our laboratory have demonstrated deficiencies in sialic acid, galactose, and N-acetylglucosamine in vWF purified from patients with type IIb von Willebrand's disease (vWD).[30,33] This suggests that the inhibited platelet adherence we have observed with AS-vWF and AS,AG-vWF may have clinical relevance. Sakariassen et al showed that infusion of 1-deamino (8-D-arginine) vasopressin (DDAVP) into type IIb vWD patients caused reduced platelet adherence to subendothelium, presumably due to the release of increased amounts of their qualitatively abnormal vWF into the plasma.[5] Although the carbohydrate composition of the patients' vWF was not described, the data of Sakariassen et al support our findings that qualitatively abnormal vWF can impair platelet adherence to subendothelium. Based on our current observations and our previous

Table 3. Effect of AS-vWF on Platelet Adherence With Reconstituted Afibrinogenemic Blood

Protein Added to Blood	Percentage of Contact (C)	Percentage of Spread (S)	Total Platelet Adherence (C + S)	Percentage Postperfusion Platelet Count Reduction
4 μg/mL vWF (n = 2)	5.69	40.56	46.25	14.8
4 μg/mL AS-vWF (n = 2)	4.25	23.35*	27.60†	19.6

Reconstituted perfusates were prepared from washed normal red cells, washed normal platelets; and citrated PPP from a patient with congenital afibrinogenemia (hematocrit, 40%; platelet count, 246,000/μL). The specified amounts of native plasma vWF or AS-vWF were added to the samples before perfusion at 37 °C for five minutes over de-endothelialized human umbilical artery segments at a wall shear rate of 2,600 sec^{-1}. Mean values are shown and the indicated values are significantly different between the two groups.

*$P < .02$

†$P < .05$.

studies of vWD, it is possible that some vWD patients show impaired primary hemostasis as a consequence of inhibited platelet adherence due to carbohydrate deficiency or other structural defects of their plasma vWF.[30,33]

Our findings suggest that the ability of plasma vWF to support platelet adherence to subendothelium requires preservation of the sialic acid content of the glycoprotein. By contrast, the RCof activity of vWF is enhanced by 20% to 40% by the removal of the negatively charged sialic acid residues.[15] This discrepancy between vWF function in primary hemostasis and the RCof assay is reflected in the clinical observation that some patients with vWD show RCof levels that fail to correlate with their bleeding times and hemorrhagic diatheses.[5-10] It further suggests that concepts regarding structure-function relationships in vWF must be tested in other systems in addition to the RCof assay to ensure their physiologic relevance.

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Conversion of the Active to Latent Plasminogen Activator Inhibitor From Human Endothelial Cells

By Eugene G. Levin and Lydia Santell

The plasminogen activator inhibitor from human endothelial cells (PAI-1) exists in two forms in the culture medium: an active form that binds to and inactivates plasminogen activators and a latent form that in its native state has no anti-activator activity. Inhibitor activity associated with the latent form can be generated by treatment with protein denaturants and makes up more than 98% of the total inhibitor activity in conditioned medium. Plasminogen activator inhibitor activity is also found in cell cytosol. This inhibitor activity is stable to SDS-treatment but is not enhanced by it. We investigated the relationship between this active cell-associated inhibitor and the latent PAI-1 found in the conditioned medium. Both intracellular and extracellular inhibitors were immunoprecipitated by a monoclonal antibody produced against the latent inhibitor from HT1080 fibrosarcoma cells and electrophoresis on SDS gels of various acrylamide concentrations demonstrated that both forms had the same M_r. Incubation of cytosol inhibitor at 37°C resulted in a decline in inhibitor activity with a half-life of ~4 hours, a rate of decline similar to that of the active PAI-1 in conditioned medium, with less than 10% of the original activity present after eight hours. This decline is accelerated at higher temperatures and is not affected by the presence of a variety of protease inhibitors. Approximately 90% of the activity can be regenerated after SDS treatment suggesting that the cell associated inhibitor, during incubation at 37°C, converts to a form similar to that found in conditioned medium. Despite these similarities, the apparent Stoke's radii of the active intracellular inhibitor and the latent inhibitor in conditioned medium were significantly different with values of 2.77 nm and 2.40 nm for active and latent PAI-1, respectively. Incubation of the active form at 37°C resulted in the shift of the Stoke's radius to that similar to the latent PAI-1 (2.45 nm). Thus, the active and latent PAI-1, while being immunologically similar and of the same apparent M_r, can be differentiated by their behavior on gel permeation columns. This suggests that the intracellular inhibitor is a precursor to the latent form.

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A SPECIFIC PROTEIN inhibitor of tissue plasminogen activator (tPA) and urokinase has been described in plasma, platelets, and a variety of cultured cells, including human endothelial cells.[1-13] This inhibitor (PAI-1) is immunologically distinct from other inhibitors of plasminogen activators such as PAI-2, which inhibits urokinase but is inefficient against tPA,[14,15] and protease nexin, which inhibits several serine proteases in addition to tPA and urokinase.[16] PAI-1 combines rapidly with plasminogen activators with a second order rate constant of $\sim 10^7 \mathrm{mol/L^{-1}s^{-1}}$[17,18] and forms an equimolar, SDS and heat stable complex that has similar properties to other protease-α_1-proteinase inhibitor complexes.[19-21] Inhibitor release from cultured cells can be stimulated with dexamethasone,[1] endotoxin,[9,22] and thrombin.[23] The rate of release of tissue plasminogen activator and its inhibitor occur independently of each other, and can be controlled separately in response to prevailing environmental conditions.[24]

The PAI-1 inhibitor is a glycoprotein with a reported M_r of ~46,000 and a pI of 4.5 to 5.[25] It has been identified in two immunologically similar forms, an active form that binds to and inhibits plasminogen activators and a latent form that has no anti-activator activity in its native state,[4,26] but can acquire anti-activator activity after treatment with protein denaturants such as SDS, urea, guanidine hydrochloride, or ammonium thiocyanate.[27] Although both latent and active forms are found in the culture medium of human endothelial cells,[28] HT-1080 fibrosarcoma cells,[4] and HTC hepatoma cells,[29] the latent form is in excess. The ratio of latent to active PAI-1 in conditioned medium is dependent on the duration of culture incubation. Conditioned medium prepared for four hours contains latent PAI-1 activity 12 times higher than active PAI-1 activity while by 8, 16, and 24 hours the ratio increases to 30, 40, and 55 times, respectively.[28] Studies with human endothelial cells indicate that inhibitor activity is also present in cell homogenates.[28] The incubation of this inhibitor at 37°C results in a decline in inhibitor activity that can be regenerated by SDS treatment. Thus, we have suggested that this inhibitor is synthesized and released active and then converts to a latent form during or after release. In this report we present studies on the relationship between the intracellular active and the extracellular latent inhibitor activity in endothelial cells and provide evidence supporting a precursor-product relationship.

MATERIALS AND METHODS

Materials. Tissue culture medium was purchased from MA Bioproducts (Walkersville, MD) and methionine-free RPMI 1640 and fetal calf serum from Irvine Scientific (Irvine, CA). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) supplies, the Bio-Sil TSK-125 HPLC gel filtration column (300 mm × 7.5 mm) and Bio-Sil TSK guard column (75 × 7.5 mm) were from Bio-Rad (Richmond, CA); Ultrogel AcA 54 from LKB (Gaithersburg, MD); Protein A-Sepharose from Pharmacia (Piscataway, NJ); N-ethylmaleimide, diisopropylfluorophosphate, phenylmethylsulfonyl fluoride, pepstatin A, leupeptin, Triton X-100, and heparin from Sigma

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Chemical Co (St. Louis); fibrinonogen (thrombin and plasminogen-free) from Calbiochem (La Jolla, CA); ^{35}S-methionine (>800 Ci/mmol) from Amersham-Searle (Arlington Heights, IL), agarose (low gelling temperature) from Miles Scientific (Naperville, IL); and EN^{3}HANCE from New England Nuclear (Boston). Human alpha-thrombin was a gift from Dr J.W. Fenton II (New York Department of Health, Albany) and had a specific activity of 2,950 U/mg. tPA (specific activity = 272,000 IU/mg) was a gift from Dr D. Collen (University of Leuven, Belgium) and was isolated from melanoma cell cultures as described.[30] The monoclonal antibody against the plasminogen activator inhibitor (PAI-1) from HT-1080 fibrosarcoma cells was a gift from Drs L.S. Nielson and K. Danø (University of Copenhagen, Denmark). Plasminogen was prepared from human plasma by affinity chromatography as described.[31]

Cell culture. Endothelial cells were isolated from human umbilical cord vein as previously described[32] and were cultured on 75 cm^2 flasks coated with 20 mg/mL calf skin gelatin. Cells were grown in RPMI 1640 containing 20% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 10 μg/mL endothelial cell growth factor (ECGF), and 90 μg/mL heparin. To prepare conditioned medium, confluent cultures were washed twice with RPMI 1640 and incubated in the absence of serum for 24 hours in 5 mL of RPMI 1640. The medium was centrifuged at 600 x g to remove cell debris and made 0.01% with Tween-80. To reduce the level of active inhibitor activity in conditioned medium, samples were concentrated ten-fold and incubated at 37°C for ten hours. After this procedure active PAI-1 activity was less than 6×10^{-4}% of the total inhibitor activity (active and latent) in 24-hour conditioned medium. Samples were frozen at −20°C until used.

Cell extracts were prepared as follows: cell monolayers were washed twice with cold 0.01 mol/L sodium phosphate, pH 7.4, 0.15 mol/L NaCl, 1 mmol/L $CaCl_2$, and 0.5 mmol/L MgCl phosphate buffered saline (PBS), scraped with a rubber policeman, and pelleted by centrifugation at 600 x g. Cell pellets were washed two more times with cold PBS followed by centrifugation at 600 x g and suspended in 0.01 mol/L sodium phosphate, pH 7.2, 0.015 mol/L NaCl (100 μL/4×10^6 cells) for ten minutes at 4°C. Cells were disrupted by homogenization until whole cells were no longer apparent by microscopic examination. The large cell fragments and nuclei were removed by centrifugation at 600 x g for ten minutes and the sample was centrifuged at 35,000 x g for 30 minutes at 4°C. Cell homogenates were made 1X PBS and stored at −70°C. This material is referred to as cell cytosol.

^{35}S-methionine labeled cell extract and conditioned medium were prepared by washing cultures two times with methionine-deficient RPMI 1640 and incubating the cells in 100 μCi/mL ^{35}S-methinone containing methionine deficient medium for six hours. The cell monolayer was washed three times with cold PBS and extracted with 1.0 mL 0.5% Triton X-100 in PBS.

Assay of fibrinolytic activity and inhibitor activity. Fibrinolytic activity was assayed on ^{125}I-fibrin coated multiwell tissue culture dishes containing in 1 mL: 4 μg human plasminogen, 0.1% gelatin, 0.1 mol/L Tris-HCl, pH 8.1, 2% Triton X-100, and tPA.[33] The rate of fibrinolysis was determined by removing an aliquot of reaction mixture from the surface of the dish and measuring the amount of radioactivity released with respect to incubation time. The results are an average of duplicate samples.

PAI-1 activity was measured as previously described.[28] Increasing volumes of inhibitor containing sample (0 to 150 μL) were added to 0.5 mL centrifuge tubes containing 0.1 mol/L sodium phosphate, pH 7.2, 2% Triton X-100 (final volume 400 μL/tube) and tPA (0.12 IU) was added to each tube. The samples were mixed and incubated for ten minutes at 37°C, and duplicate aliquots (200 μL) from each tube were added to ^{125}I-fibrin coated wells containing 750 μL of assay buffer (0.1 mol/L Tris-HCl, pH 8.1, 0.1% gelatin, 2% Triton X-100). Plasminogen (50 μL) was added and the assay was incubated at 37°C. The assay was terminated after 40% to 50% of the ^{125}I counts were removed from control wells not containing inhibitor (about one hour). Percent residual activity *v* inhibitor sample volume was plotted on a linear graph, and the number of inhibitory units in each sample was calculated from the volume of sample giving 50% inhibition of tPA activity (equal to 0.03 IU).[4,28] If necessary, samples were diluted in phosphate-Triton X-100 buffer to insure that 50% tPA inhibition occurred within the volumes used. One unit of inhibitor activity is that neutralizing one international unit of tPA activity.

Inhibitor samples were prepared for assay as follows: To measure latent inhibitor activity SDS was added to samples to produce a final concentration of 0.2% and the mixture was incubated at 37°C for ten minutes. The samples were made 2% with Triton X-100 and then added to the assay tubes. Samples to be measured for active inhibitor activity were made 2% with Triton X-100 only and assayed. Latent inhibitor activity was calculated by subtracting inhibitor activity in nondenatured samples (active) from that measured in SDS-treated (active and latent) samples. SDS does not interfere with active inhibitor activity.[28] The presence of SDS and Triton X-100 had no effect on tPA activity. Intra-assay variation was less than 10%.

To determine the effect of protease inhibitors on the decline of active PAI-1 activity during 37°C incubation, 100 μL of cell cytosol was incubated at 37°C for four hours in the presence or absence of each protease inhibitor at the final concentration indicated. All samples were dialyzed for 16 hours against PBS at 4°C and assays were performed to determine the level of PAI-1 activity remaining after incubation.

Gel filtration. Gel filtration studies were performed using Ultrogel AcA 54 columns (1.0 × 45 cm), equilibrated in 0.1 mol/L sodium phosphate, pH 7.2, 0.25 mol/L NaCl, 0.01% Tween-80 at 4°C using descending flow at 9 mL/hr. Samples (250 μL) contained conditioned medium (5X concentrated) or cytosol and calibrating proteins. The column was eluted with the equilibration buffer and 0.5 mL fractions collected. Protein was monitored by absorption at A_{280}. Blue dextran and sodium azide were used as internal markers for the determination of void volume (V_0) and total volume (V_t), respectively. Calibrating proteins and their Stoke's radius (a) were albumin, a = 3.6 nm; β-lactoglobulin, a = 2.7 nm; myoglobin, a = 1.8 nm; and cytochrome C, a = 1.6 nm. The values of (a) for each protein were calculated from the diffusion coefficient according to the equation $a = kT/6\pi\eta D_{20,w}$, where k is the Boltzman's constant, T is 293°K, η is the viscosity of H_2O at 20°C, and D is the diffusion coefficient corrected to H_2O at 20°C. Diffusion coefficients were obtained from the Handbook of Biochemistry.[34]

High performance gel permeation chromatography. Chromatography was performed on a Waters Model ALC-204 liquid chromatograph using a Bio-Sil TSK 125 column [7.5 × 300 mm column (M_r range = 500 to 60,000)], protected by a 7.5 × 75 mm Bio-Sil guard column, and equilibrated with 0.1 mol/L sodium phosphate, pH 7.2, 0.25 mol/L NaCl. Cytosol (100 μL) or conditioned medium (10X concentrated, 100 μl) was applied and 200 μL fractions were collected and assayed for the presence of PAI-1 activity. The column was calibrated using ovalbumin, urokinase, and soybean trypsin inhibitor. The flow rate for all determinations was 0.5 mL/min and the column effluent was monitored at A_{280}.

Polyacrylamide gel electrophoresis. SDS-PAGE was performed by the procedure of Laemmli[35] with resolving gels as stated (see text) and stacking gels of 4% acrylamide. M_r standards included phosphorylase B (92,500), bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (31,000), and soybean trypsin inhibitor (21,500). Samples (100 μL) were boiled in 3% SDS and 5% β-mercaptoethanol for three minutes before application. Gels to be analyzed by autoradiofluorography were stained with 0.1% Coomassie brilliant blue R-250 and 50% trichloracetic acid, destained in 10% acetic acid and then treated with an autoradiography enhancer (EN^{3}HANCE) for one hour followed by 30 minutes in H_2O. Gels were dried and placed on x-ray film (XR-5, X-OMAT R film) at −70°C. Gels to be analyzed for inhibitor activity were soaked in 5% Triton X-100 for 1.5 hours, dried with a paper towel, and placed on a reverse fibrin autograph gel.

Reverse fibrin autography. Plasminogen activator inhibitor in SDS-polyacrylamide gels was detected by layering the gel previously soaked in 5% Triton X-100 on an agarose gel containing 1% agarose, 0.057 NIH U/mL thrombin, 2 mg/mL fibrinogen, 27 µg/mL plasminogen, 1% Triton X-100 and 0.56 IU/mL tPA. The agarose gels were prepared by the method of Erickson et al.[36] Briefly, 2% agarose (low gelling temperature) heated to 45°C was added to 45°C PBS containing the thrombin, plasminogen, and tPA. After mixing, the fibrinogen was added and the mixture layered onto a glass plate. Following agarose solidification and fibrin formation, the acrylamide gel was placed on top of the agarose fibrin matrix and then incubated at 37°C until general lysis had occurred (~3 hours) Opaque lysis resistant zones indicated the presence of protease inhibitors in the polyacrylamide gel.

Immunoprecipitation. Immunoprecipitation of PAI-1 was performed with a monoclonal antibody (IgG_1 subclass) produced against the plasminogen activator inhibitor from HT-1080 fibrosarcoma cells[37] and designated clone 1. To precipitate ^{35}S-methionine labeled inhibitor, 5 mL conditioned medium and 1 mL cytosol samples were dialyzed against 0.1 mol/L Tris-HCL pH 8.0 and prepared with 0.1% albumin, 1 mmol/L PMSF, 100 µ/ml trasylol, and either 5 µg of antiplasminogen activator inhibitor antibody or pre-immune mouse IgG was added. The mixture was incubated for 16 hours at 4°C and immune complexes precipitated by the addition of 50 µL of a protein A-Sepharose suspension (50% beads). Sepharose beads were removed by centrifugation for two minutes at 13,000 x g and were washed three times with 50 mmol/L Tris-HCl, pH 7.2, 0.1% SDS, 0.5% NP_{-40}, and 0.1% sodium deoxycholate, and one time with H_2O.

RESULTS

Active and latent inhibitor in endothelial cell cultures. We have previously demonstrated that the intracellular plasminogen activator inhibitor activity in endothelial cells is not enhanced by treatment with SDS and therefore does not contain latent inhibitor.[28] In contrast only a small fraction of the inhibitor in conditioned medium was active (<2%) and incubation of the medium at 37°C reduced the amount of detectable inhibitor activity even further. We have now determined that active PAI-1 activity can be reduced to less than $6 \times 10^{-4}\%$ of total inhibitor activity detected after SDS treatment following incubation of conditioned medium at 37°C for ten hours in the absence of cells (data not shown). Therefore, essentially homogeneous samples of latent PAI-1 and active inhibitor can be obtained from human endothelial cell cultures.

We have examined the immunochemical similarity between the intracellular inhibitor and the extracellular latent PAI-1 using a monoclonal antibody developed against the latent form of the inhibitor purified in the absence of denaturants. Immunoprecipitation of ^{35}S-methionine-labeled cytosol and conditioned medium showed single specific bands of radioactivity migrating to identical positions on SDS gels (Fig 1). Reverse fibrin autography of the immunoprecipitates showed a single lysis resistant zone at a position identical to the radioactive bands and to those observed in lanes containing crude samples of conditioned medium and cytosol (Fig 2).

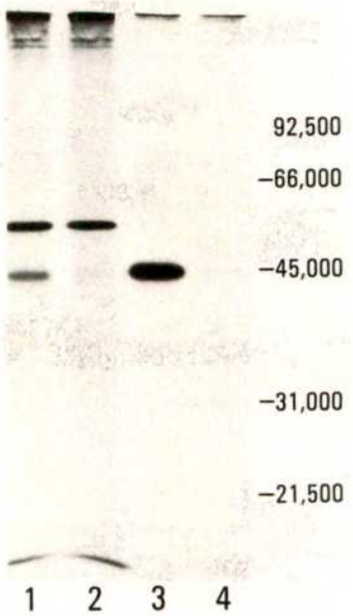

Fig 1. Immunoprecipitation of the plasminogen activator inhibitors from cytosol and conditioned medium. Endothelial cell cultures were labeled with 100 µCi/mL ^{35}S-methionine for six hours, the conditioned medium was removed, and the cells extracted with 1 mL 0.5% Triton X-100 in PBS. Conditioned medium was incubated for ten hours at 37°C before addition of antibody. Monoclonal antibody against PAI-1 (5 µg) or 5 µg of pre-immune mouse IgG was added to 1 mL of conditioned medium and cell extract and the immune complexes were precipitated under conditions described in Materials and Methods. Immunoprecipitates were analyzed under reducing conditions on 10% SDS-polyacrylamide gels and autoradiofluorography. The positions of the M_r markers are indicated to the right. Lanes 1 and 2, active PAI-1 (cytosol) treated with anti-inhibitor antibody and pre-immune IgG, respectively; lanes 3 and 4, latent PAI-1 (conditioned medium) treated with anti-inhibitor antibody and nonimmune IgG, respectively.

Conversion of the active to latent inhibitor in cell cytosol. Cell cytosol was incubated at 37°C for 0, 2, 4, 6, and 8 hours and active inhibitor activity was measured for each time point (Fig 3). Inhibitor activity declined in a linear fashion with a half-life of close to four hours and only 10% of the starting activity was detected after eight hours. To determine whether time of incubation affected the regeneration of activity by SDS treatment, samples incubated at 37°C for various times were treated with 0.2% SDS and the level of inhibitor activity compared with samples remaining at 4°C. (No decline in activity is seen after eight hours at 4°C.) Including all time points an average of 90% of the original activity (90.4% ± 7.5%, 14 determinations) could be recovered after SDS treatment with values ranging from 83% to 100% of starting activity. In individual determinations greater than 90% recovery was attained in eight of the samples while the rest showed recoveries of 83% to 90%. However, no correlation between the time of incubation and the activity recovered was observed.

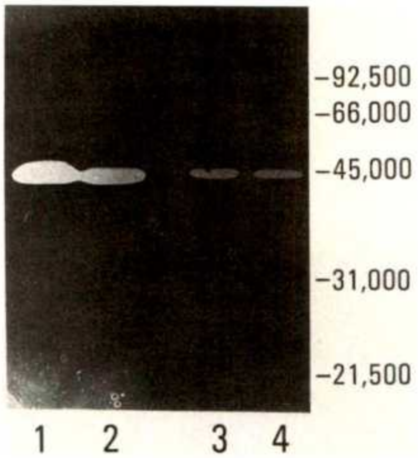

Fig 2. **SDS-PAGE and reverse fibrin autography of cell cytosol and conditioned medium and the anti-PAI-1 immunoprecipitates. Cytosol (100 μL) and 24-hour conditioned medium (200 μL) previously incubated for ten hours at 37°C in the absence of cells were fractionated in a 10% SDS-polyacrylamide gel under reducing conditions and the inhibitor activity visualized by reverse fibrin autography as described in Materials and Methods. Lane 1, cytosol; lane 2, conditioned medium. Cell extracts and conditioned medium were immunoprecipitated with anti-PAI-1 described in Materials and Methods and immune complexes removed with 50 μL protein A-Sepharose. The Sepharose beads were washed two times with 1 mL PBS, suspended in 3% SDS-5% β-mercaptoethanol and boiled for three minutes. Samples were analyzed by SDS-PAGE and reverse fibrin autography. Lane 3, active PAI-1; Lane 4, latent PAI-1.**

We have also compared the efficiency of immunoprecipitation of inhibitor in cytosol before and after 37°C incubation for eight hours with the anti–PAI-1 monoclonal antibody. Monoclonal antibody (2.5 μg) was added to the cytosol and the residual inhibitor activity was measured after precipitation. In both cases ~70% (67% for preincubated and 65% for postincubated; three determinations) of the starting inhibitor was precipitated by the antibody.

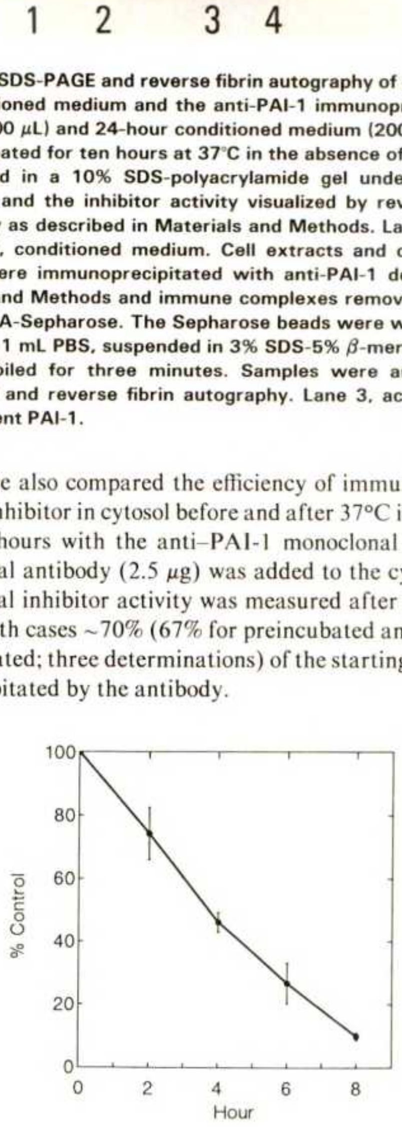

Fig 3. **Decrease of intracellular plasminogen activator inhibitor activity at 37°C. Cell cytosol (400 μL) was incubated at 37°C and aliquots removed at 2, 4, 6, and 8 hours and stored at 4°C. Samples were assayed simultaneously as described in Materials and Methods and the level of tPA inhibition reported as a percent of the inhibitor activity in samples remaining at 4°C. Data points represent the mean ± SD of at least three determinations.**

The effect of higher temperatures on the loss of active inhibitor was also assessed. Following incubation for two hours at 56°C, <10% of control activity remained while incubation at 70°C resulted in a complete loss of activity. This is in comparision to the >70% activity remaining at 37°C at two hours. To determine whether higher temperatures irreversibly inactivated the inhibitor, SDS treatment was performed. The recovery achieved after 56°C was 87%, similar to that observed at 37°C, but only 63% after 70°C incubation (4 determinations each).

The possibility that the loss of activity in the cytosol results from proteolysis by either a cell-associated protease or a serum contaminant was also investigated (Table 1). We attempted to block the decline in inhibitor activity in cell cytosol by incubating samples at 37°C in the presence of PMSF (1 mmol/L), EDTA (5 mmol/L), N-ethylmaleimide (15 mmol/L), pepstatin A (10 mmol/L), DFP (1 mmol/L) and leupeptin (10 mmol/L). Samples were assayed and inhibitor activity compared with samples incubated at 37°C in the absence of protease inhibitor. In no case after four hours incubation was the level of inhibitor activity significantly different than that of the 37°C control.

Gel filtration. High performance gel filtration chromatography of the cytosol and conditioned medium was carried out and fractions analyzed for active and latent inhibitor, respectively. The elution patterns of the two forms shown in Fig 4 are clearly distinguishable. Analysis of the cell cytosol fractions in the absence of SDS treatment showed a single peak of activity eluting between ovalbumin and urokinase. No additional inhibitor activity was observed after treatment with SDS. The latent inhibitor activity in conditioned medium eluted more slowly, after urokinase. Conventional gel filtration chromatography on Ultrogel AcA 54 showed similar results (Fig 5). Fractionation of cell cytosol showed two peaks of inhibitor activity, a small peak eluting between the void volume and albumin and a major peak eluting slightly ahead of β-lactoglobulin. Again, treatment of column fractions with SDS had no effect on the elution profile of the cytosol inhibitor. The latent inhibitor also fractionated into two peaks similar in nature to that observed with the active inhibitor. Both peaks eluted after their cytosol inhibitor counterparts and the major latent inhibitor activity peak was not observed for several fractions following β-lactoglobulin.

Table 1. Effect of Various Protease Inhibitors on the Decline of Cytosol Inhibitor Activity

Inhibitor	Percent 4°C Control Residual Activity	 Residual Activity With Inhibitor
EDTA (5 mmol/L)	38	43
PMSF (1 mmol/L)	43	44
N-ethylmaleimide (15 mmol/L)	41	39
DFP 1 (mmol/L)	40	43
Leupeptin (10 mmol/L)	34	40

Cytosol was prepared and incubated in the presence or absence of the protease inhibitors listed at 37°C for four hours. Residual inhibitor activity was assayed as described in Materials and Methods.

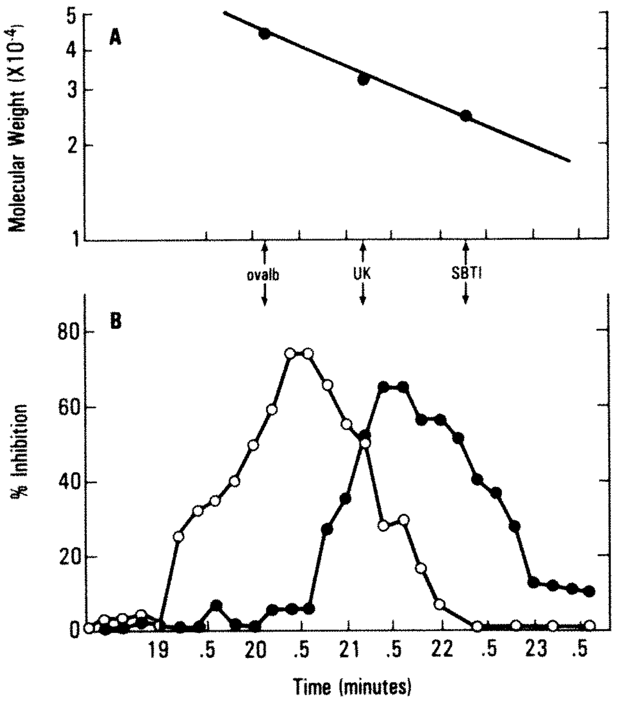

Fig 4. High performance gel permeation chromatography of active and latent PAI-1. Cytosol (100 μL) or conditioned medium (10X concentrated, 100 μL) were chromatographed on a Bio-Sil TSK-125 column (300 × 7.5 mm), equilibrated with 0.1 mol/L sodium phosphate, pH 7.2, 0.25 mol/L NaCl. The samples were eluted at a flow rate of 0.5 mL/min, and 0.2 mL fractions were collected. Fractions were assayed in the presence or absence of SDS, as described in Materials and Methods. (A) Standard curve defined by the calibrating proteins ovalbumin (ovalb), urokinase (UK) and soybean trypsin inhibitor (SBTI). Calibrating proteins were run under identical conditions to the experimental samples. (B) Column elution profile of the active (○—○) and latent (●—●) plasminogen activator inhibitors. Each data point is the average of corresponding fractions from two different experiments.

The apparent Stoke's radius of the two forms represented by the two major peaks of inhibitor activity were determined from a standard curve of $\sqrt{-\log K_d}$ *vs* the Stoke's radii of the standard proteins used (Fig 6).[38] The Stoke's radius of the active inhibitor was 2.77 ± 0.09 nm and the latent inhibitor was 2.40 ± 0.02 nm. Similar values were obtained when a standard curve of K_d *vs* Stoke's radius was used.

To determine whether a change in the Stoke's radius of the active inhibitor to that of the latent inhibitor occurred following the loss of inhibitor activity, cytosol was incubated at 37°C for eight hours (Fig 3), chromatographed, and the inhibitor activity profile in the fractions compared with a duplicate sample left at 4°C (Fig 5). The sample incubated at 37°C showed a shift in the major peak to a position corresponding to that of the latent inhibitor in conditioned medium with a determined Stoke's radius of 2.45 nm (two determinations: 2.47 and 2.43). A smaller peak of inhibitor activity eluting at the position of the active inhibitor activity was also observed. This presumably represents active inhibitor that was not converted to the latent form during incubation. Thus, during incubation at 37°C, a shift in the elution profile of active PAI-1 to that identical to the latent PAI-1 occurs concomitantly with a loss of activity. In addition, a shift back to the elution profile characteristic of the active form was induced by treating the incubated cytosol inhibitor or latent inhibitor with guanidine HCl and chromatographing it over the same column (Fig 7).

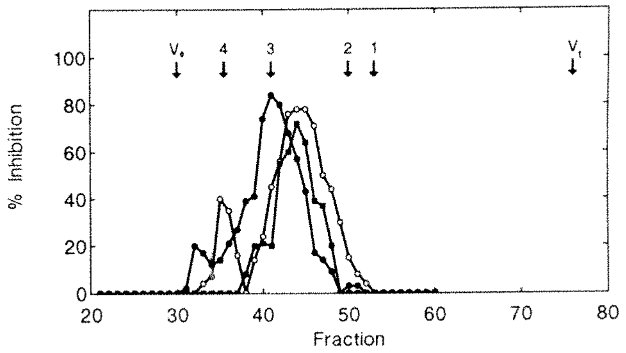

Fig 5. Gel filtration chromatography of active and latent PAI-1 in Ultrogel AcA 54. Cytosol (250 μL) and conditioned medium (5X concentrated, 250 μL) were chromatographed on an Ultrogel AcA 54 column (1 × 45 cm) equilibrated with 0.1 mol/L sodium phosphate, pH 7.2, 0.25 mol/L NaCl, 0.01% Tween 80. The column was eluted at 9 mL/hr and 0.5 mL fractions were collected. Fractions were assayed as described in Materials and Methods. The designated markers are: V_t, total volume; 1, cytochrome C; 2, myoglobin; 3, β-lactogloblin; 4, bovine serum albumin; V_o, void volume. (●—●), active; (○—○), latent; (■—■), active incubated at 37°C for eight hours.

Mass estimation by electrophoretic mobility. While the behavior of the two inhibitor forms on SDS-polyacrylamide gels indicates that both have the same molecular weights, their behavior on gel permeation columns suggests differences in the molecular properties of these two inhibitor forms. The molecular weights that would be predicted by this technique are ~40,000 for the active inhibitor and ~33,000 for the latent inhibitor (Fig 4). However, molecular weight determination by this technique is subject to error if the molecular properties of the proteins studied are not consistent with those of the reference proteins used to establish the standard curve.[39] Absorption effects or differences in the

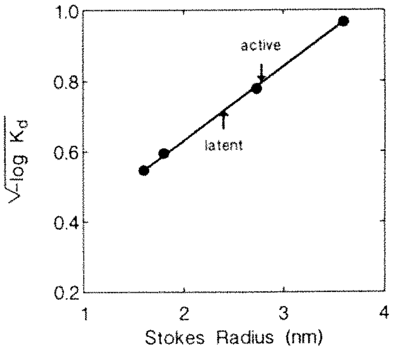

Fig 6. Determination of Stoke's radius of active and latent PAI-1. A standard curve was constructed from $\sqrt{-\log K_d}$, where K_d is the distribution coefficient *v* Stoke's radius of the calibrating proteins. (●), calibrating enzymes (left to right): cytochrome C, myoglobin, β-lactoglobulin, bovine serum albumin.

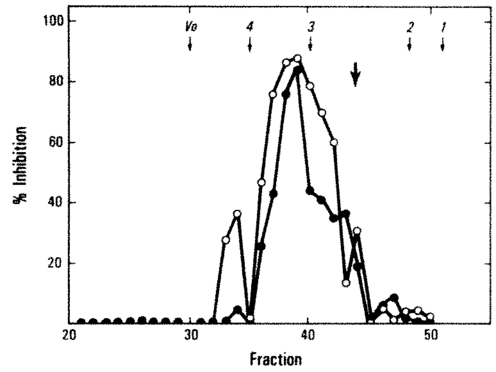

Fig 7. Gel filtration chromatography of guanidine HCl treated latent and 37°C-treated cytosol PAI-1 in Ultrogel AcA 54. Cytosol was incubated at 37°C for eight hours, treated with 4 mol/L guanidine HCl for four hours, and dialyzed in PBS overnight. Conditioned medium was treated in an identical manner and treated cytosol (○—○) or 10× conditioned medium (●—●) (250 μL each) were chromatographed as described in Fig 5. The large arrow identifies the position of the latent PAI-1.

shape of the proteins may create anomalous behavior on gel permeation chromatography.[38] However, neither can it be assumed that the behavior of these two inhibitor forms on SDS-gels accurately reflects their molecular mass. Molecular weight estimation on polyacrylamide gels under a single set of conditions also may be in appreciable error if the SDS-binding properties of the proteins are not consistent with the reference proteins.[40] SDS-binding properties may be investigated and a more accurate estimation of the mass of the protein can be determined by electrophoresis of the samples in separate gels of various polyacrylamide concentrations.[40,41] Thus, to insure that the results in Figs 1 and 2 reflected the true mass of both forms, the mobility of both active and latent inhibitor was analyzed in five separate gels with polyacrylamide concentrations of 7% to 11% and the charge density and M_r compared. Plots of relative mobility of standard proteins (phosphorylase B, transferrin, albumin, ovalbumin, and soybean trypsin inhibitor) v polyacrylamide concentration were linear and yielded identical verticle intercepts (extrapolation to electrophoresis in 0% acrylamide) indicating similar SDS binding properties and therefore similar charge densities (Fig 8). Atypical SDS binding properties relative to the standard proteins would result in a lower vertical intercept. However, in all gels used, both active and latent inhibitor migrated to the same relative position, and the verticle intercepts of the two PAI-1 forms were identical and corresponded to that of the reference proteins. Thus, the charge density of the two inhibitor forms in SDS are consistent with the standard proteins and the migration rate on gels of any polyacrylamide concentration should be an accurate reflection of the molecular mass of the inhibitors. The position of inhibitor activity for SDS-gels of 8%, 9%, and 10% acrylamide are shown in the inset to Fig 8.

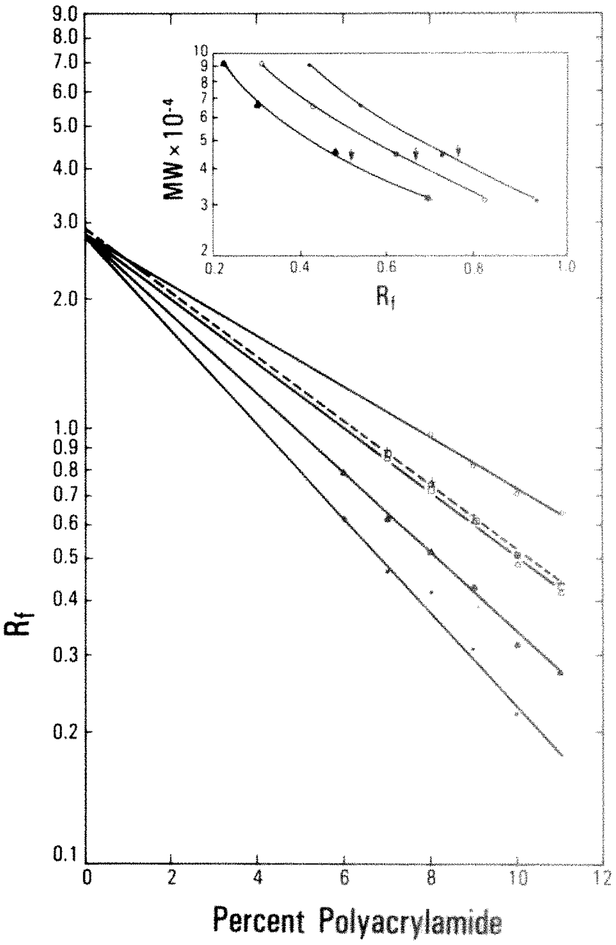

Fig 8. Electrophoretic mobility in SDS of the active and latent PAI-1 in varying concentrations of polyacrylamide. Samples of cytosol (100 μL) or conditioned medium (100 μL) were fractionated under reducing conditions according to Laemmli[35] in gels of various polyacrylamide concentrations. The relative mobility of the proteins (R_f) is plotted v polyacrylamide concentrations for phosphorylase B (●—●), bovine serum albumin (▲—▲), ovalbumin (△—△), and soybean trypsin inhibitor (○—○). Active (+—+) and latent (□—□) inhibitor activity was detected by reverse fibrin autography as described in Materials and Methods. All proteins studied have normal SDS binding properties as indicated by the essentially identical vertical intercept. *Inset:* Determination of the M_r of the inhibitor from three separate SDS gels of 8% (●—●), 9% (○—○), and 10% (△—△) acrylamide.

DISCUSSION

Human endothelial cells produce an inhibitor of plasminogen activators (PAI-1) immunologically similar to the inhibitor found in platelets and plasma,[1-13] and in the culture medium of various cultured cells, including rat hepatoma cells,[29] HT-1080 fibrosarcoma cells,[4] Hep G2 hepatoma cells,[10] and bovine endothelial cells.[3] In most cell lines tested, it was shown that the inhibitor activity present in conditioned medium was of two immunologically related types, one that reacted with and inhibited plasminogen activators and another that was only observed after treatment with SDS or other protein denaturants.[4,10,26-29]

We have suggested that the latent form of the PAI-1 is derived from an active form following release from the cell. This proposal is based on the observations that (1) there is a cell-associated plasminogen activator inhibitor activity that is neither destroyed by nor enhanced by SDS treatment, (2) incubation of cell cytosol at 37°C in the absence of cells results in a decline in the inhibitor activity, and (3) the inhibitor activity that is reduced at 37°C can be regenerated by SDS denaturation in the same manner as the latent inhibitor in conditioned medium. We have extended these studies to more definitively establish the relationship between the cell-associated inhibitor activity and the extracellular latent PAI-1. Since cell cytosol contains only active inhibitor and conditioned medium can be depleted of active PAI-1 by incubation at 37°C (after which active inhibitor activity accounts for <0.1% of total inhibitor activity), the endothelial cell culture can be used as a source of both forms with no cross-contamination.

We have used a monoclonal antibody raised against the latent PAI-1 produced by HT-1080 cells that cross-reacts with the inhibitor in endothelial cell conditioned medium[37] to assess the immunologic similarity between the inhibitor in cell cytosol and the latent inhibitor in conditioned medium. In both cases, immunoprecipitation resulted in a single specific band on autoradiographs corresponding to identical positions on the SDS gel. SDS-PAGE and reverse fibrin autography of the precipitated immune complexes showed a single lysis resistant zone for each that corresponded to the same position, relative to the standard proteins, as the ^{35}S-methionine labeled protein. The inhibitor activity in immunoprecipitates comigrate with the inhibitor activity in untreated cell cytosol and conditioned medium. Thus, the latent inhibitor present in conditioned medium and the active inhibitor in cell cytosol are immunologically related. This monoclonal antibody will also recognize the latent inhibitor activity generated from cytosol active inhibitor during 37°C incubation and immunoprecipitate both forms to the same degree. Further analyses of the immunologic differences and similarities of the two PAI forms using monoclonal antibodies is being pursued.

We have found that the rate of decline of cytosol active inhibitor at 37°C over an eight-hour period is slightly different than the results obtained with the active inhibitor in conditioned medium.[28] In the latter case, the decline in activity was biphasic with an initial half-life of approximately two hours followed by a slower decline in activity beginning at approximately 2.5 hours and with a half-life of four hours. With cytosol, the initial rapid phase was not observed but the decline in activity was similar to the second phase suggesting that the two hour half-life is mediated by something other than that responsible for the cytosol active to latent conversion. The data presented concerning regeneration of inhibitor activity following SDS treatment suggest that the changes that occur during the decline in inhibitor activity are not always or completely reversible. In the samples tested we observed >90% recovery in only 57% of the samples with the rest between 80% and 90%. This was not SDS concentration dependent since similar results were observed with SDS concentrations up to 1% (data not shown). The even lower recovery in samples incubated at 70°C (63%) raises the possibility that the loss of activity does not necessarily represent the conversions of the active inhibitor to its SDS-recoverable form, but may include the denaturation of the molecule beyond recovery. This indicates that the use of activity measurements after SDS denaturation may not be accurate for determining total inhibitor antigen concentration based on specific activity.

While the mechanism initiating the decline in inhibitor activity is not obvious from the data presented, the physical studies performed indicate that differences in the hydrodynamic properties of the two PAI forms exist. These differences do not appear to result from a reduction in M_r due to cleavage of the polypeptide chain. SDS-PAGE of the two inhibitor forms analyzed by both autoradiography and reverse fibrin autography indicates that within the limitations of molecular weight estimations by this technique, no difference in the molecular weight of the inhibitor appears. Furthermore, the similar rate of migration of the two forms in SDS gels is not coincidental due to differing SDS-binding properties. These differences would become apparent when the cytosol and conditioned medium inhibitors are electrophoresed on gels of varying acrylamide concentration, which was not the case.[40,41] Therefore, we can conclude that the relative molecular mass of the two inhibitors as determined by this technique is accurate. In addition, we have not been able to detect a decline in the extent of inactivation at 37°C by several protease inhibitors (pepstatin A, DFP, PMSF, N-ethylmaleimide, EDTA and leupeptin), eliminating a variety of cell and serum associated proteases that might be responsible for a proteolytic cleavage leading to a loss of activity. Since molecular weight determinations on SDS gels would not detect the molecular weight change resulting from the release of a few amino acids and a protease not falling into a group inhibited by the protease inhibitors used would not be obvious, it is difficult to eliminate the possibility of cleavage. However, amino acid sequence analysis of the latent form isolated from HT-1080 fibrosarcoma cell conditioned medium demonstrates that the amino acid sequence of both the amino- and carboxy-terminal ends coincides with that predicted from PAI-1 cDNA clones.[42,43] It would be reasonable to conclude then that the similarity between the predicted and actual sequence precludes the possibility that proteolytic cleavage is responsible for the active to latent form conversion.

Even if limited proteolysis occurred, it could not entirely explain the differences in the behavior of the two forms during gel permeation chromatography. The M_r predicted by gel chromatography for active and latent forms are slightly >40,000 and ~30,000, respectively (Fig 5), a difference that would easily be detected by SDS-PAGE. However, the behavior of protein molecules on gel permeation chromatography is not necessarily a function of M_r but more appropriately determined by its shape and hydration.[39] Molecules that are not consistent with calibrating proteins with respect to shape and hydration will show anomalous behavior and erroneous apparent M_r. That is what may be occurring with the plasminogen activator inhibitor. We interpret the similarity in M_r of the active form determined from gel chromatography and SDS-PAGE (~40,000) as indicating that the hydrodynamic properties of the intracellular active form are more consistent with those of the calibrating proteins and that it is during the decline in activity that changes in the properties occur that cause aberrant hydrodynamic behavior.

The return of the elution profile of the latent form following guanidine HCl treatment to that characterizing the active form would suggest that a conformational change is responsible. On the other hand, it is possible that the difference in the elution profiles may be due to increased binding to the column matrix. If this were the case then properties of the inhibitor distinct from conformation would be responsible but would still define a difference between active and latent forms. In either case the shift in apparent Stoke's radius of the cytosol active PAI-1 after 37°C incubation to that identical with the latent inhibitor is consistent with our suggestion that the active intracellular inhibitor is a precursor to the latent form found in endothelial cell conditioned medium.

While the data presented here begin to define the change in the molecular properties accompanying the loss of inhibitor activity, the hydrodynamic and physical properties of the two forms of the inhibitor need to be studied in greater detail. More direct evidence for a difference in the shape of the molecule and the relative degree of asymmetry may explain the differences in the hydrodynamic behavior of the two molecules. Other biochemical characteristics such as glycosylation or hydrophobicity also may affect or be involved in the change.

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Recurrence of Acute Lymphoblastic Leukemia in Donor Cells After Allogeneic Marrow Transplantation Associated With a Deletion of the Long Arm of Chromosome 6

By N. Schmitz, W. Johannson, G. Schmidt, K. von der Helm, and H. Löffler

This report concerns a woman who experienced a relapse of acute lymphoblastic leukemia (ALL) associated with an interstitial deletion of the long arm of chromosome 6 in donor cells more than 4 years after allogeneic bone marrow transplantation (BMT). Direct bone marrow preparations revealed the presence of two leukemic clones 46,XY,del(6)(q23q25) and 45,X,−4,del(6)(q23q25), +8,−15,−21,+i(21q), +mar, the former clearly indicating that male donor cells were involved in the malignant process. Relapse as evidenced by these chromosome anomalies was confined to metaphases from directly prepared marrow cells and phytohemagglutinin (PHA)-stimulated peripheral blood cells. Cytogenetic analyses of T cell colonies gave predominantly normal donor karyotypes (65 of 69 mitoses) along with three host mitoses and a single donor metaphase carrying the 6q− anomaly. The marrow stroma, as represented by first-passage adherent layer cells from long-term marrow cultures, showed 17 of 19 host metaphases. One of two donor cells found within the stromal elements exhibited a 6q− chromosome. In the subsequent remission mitoses derived from myeloid and lymphoid cells were exclusively of donor origin, and chromosomal abnormalities could no longer be detected. Stromal elements remained host-derived (14 of 16 mitoses).
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RECURRENCE OF LEUKEMIA in donor cells after allogeneic bone marrow transplantation (BMT) is rare. With an estimated 10,000 marrow transplants having been performed worldwide by the end of 1984[1] only seven such cases have been reported.[2-8] Three of these patients had suffered from acute lymphoblastic leukemia (ALL) before grafting[2,3,6] and morphological examination[2,3] or cytochemistry and immunophenotyping[6] suggested that the original tumor had recurred after BMT. Furthermore, one patient with ALL[9] and three patients with acute myelogenous leukemia[10,11] have been described who developed a fatal Epstein-Barr virus-related lymphoproliferative disorder involving donor cells shortly after transplant. This entity clearly differs from recurrent leukemia with respect to the morphological appearance, the clinical course, and the immunological phenotype allowing its identification as a polyclonal[11] or monoclonal[9,10] tumor of B cell origin.

In this article we report the first case of recurrent ALL in donor cells associated with an interstitial deletion of the long arm of chromosome 6, which is held to be one of the specific chromosome abnormalities in ALL.[12] In addition to karyotyping directly prepared bone marrow cells and peripheral blood lymphocytes from the patient after stimulation with phytohemagglutinin (PHA), we also grew long-term marrow cultures (LTC), T-cell colonies, and early erythropoietic (BFU-E) and granulopoietic progenitors (CFU-C) for chromosomal studies. This gave us the opportunity to perform detailed analyses on the patient's myeloid and lymphoid compartments and marrow stroma cells to determine whether they were of host or donor origin and how they participated in the leukemic process.

Case Report

In April 1979 acute T-lymphoblastic leukemia (T-ALL, FAB L-2 classification) was diagnosed in a 22-year-old white woman. The diagnosis was based on the morphological appearance of blast cells and on the results of cytochemical staining. Seventy percent of blast forms were focally positive for acid phosphatase (Fig 1A) but lacked myeloperoxidase and naphthol-AS-D-chloroacetate esterase activity (Professor K. Lennert, Institute of Pathology, Kiel, Germany).

Treatment was begun with vincristine, daunorubicin, and prednisone for three weeks with no response. The patient was then treated in accordance with the protocol of the German multicenter ALL/AUL study group,[13] with some modifications. She received vincristine, adriamycin, L-asparaginase, and dexamethasone for four weeks, followed by a combination of cytosine arabinoside, 6-mercaptopurine (6-MP), intrathecal methotrexate (MTX), and cranial irradiation (18 Gy). A remission was induced and the patient was maintained on 6-MP and MTX until consolidation therapy[13] was administered in October 1979.

Maintenance therapy was continued until October 1981, when a deteriorating platelet count heralded relapse. The diagnosis of T-ALL was confirmed. In addition to the typical staining pattern for acid phosphatase, the blast cells were also positive for dipeptidylaminopeptidase IV, which is a highly specific marker for T cells at an early stage of differentiation.[14] A second remission was achieved by reinstituting the German ALL/AUL study protocol.

After a conditioning regimen including 12 mg MTX on days −9 and −3, 60 mg/kg cyclophosphamide on days −5 and −4, and 10 Gy total body irradiation on day −1, the patient received an allogeneic BMT from her HLA-identical brother in May 1982. In order to prevent graft-*v*-host disease (GVHD), T lymphocytes were removed from the donor marrow using a polyclonal antihuman T-cell globulin,[15] and MTX was administered until day +102.[16] Engraftment was prompt and the further posttransplant course was uneventful except for a mild skin rash not clearly attributable to GVHD, which spontaneously resolved.

On day +878 after BMT a second relapse (first relapse after BMT) occurred. The morphology of the leukemic cells was unchanged when compared with slides from the time of the initial diagnosis or the time of relapse before BMT. Terminal deoxynucleo-

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tidyl transferase was positive. Application of the initial treatment regimen was again successful, and a complete remission was verified by bone marrow aspiration.

The patient continued in remission until day +1464, when granulocytopenia and thrombocytopenia developed. The marrow examination on day +1485 revealed 87% blast cells. Morphologically, the blast forms were of lymphoid appearance. They stained positive for PAS (84%), but this time were negative for acid phosphatase (Fig 1B) as well as for myeloperoxidase and nonspecific esterase. The patient was treated with cytosine arabinoside (1.0 g/m^2 every 12 hours for six consecutive days) and mitoxantrone (10 mg/m^2 daily for three days). This therapy was tolerated well, and a fourth complete remission was attained. At the time of this writing the patient has stayed in unmaintained remission for six months and is currently leading a normal life.

The patient was advised of the BMT and chemotherapy procedures and the attendant risks in accordance with institutional guidelines, and gave informed consent.

MATERIALS AND METHODS

Blood and bone marrow specimens. Peripheral blood and bone marrow cells obtained after informed consent were collected in 1 mL of alpha medium containing 800 U of preservative-free heparin. In addition to BFU-E and CFU-C, which were grown only from the remission marrow, stromal elements as represented by passaged adherent cells of marrow LTC, T-lymphocyte colonies, and direct preparations of bone marrow metaphases and of unseparated peripheral blood cells stimulated with PHA were all karyotyped at the time of second relapse after BMT (day +1485) and six weeks after the ensuing complete remission had been achieved (day +1557).

Long-term cultures. Initiation and maintenance of long-term marrow cultures and the procedure for detachment of the adherent cell layer followed a protocol described by Coulombel et al.[17] When the adherent layer had grown to confluence (29 days after initiation of cultures from the patient's marrow at relapse and after 27 days of culture of the remission marrow) the nonadherent cells and all of the growth medium were removed from the culture dishes. After trypsinization of the adherent cell layer,[17] the cells were washed twice, suspended in fresh long-term medium, and transferred to new culture dishes. After two additional days of culture most cells had attached to the bottom of the dish, assumed an angulated to spindle-shaped appearance, and were rapidly dividing, as judged by a steady increase in the number of cells per microscopic field. At that stage, cultures were terminated and processed for cytogenetic analysis.

Hematopoietic colony assays. BFU-E[18] and CFU-C[19] were assayed as described with minor modifications. BFU-E were plucked for cytogenetic analysis as early as day 11 and CFU-C on day 7, in order to make sure that a significant number of proliferating cells was present.

T-lymphocyte colony assay. A modification of an assay developed by Aye and Dunne[20] was used. Ficoll-separated peripheral blood cells (final concentration 5×10^5 cells/mL) were plated in 35-mm Petri dishes containing 15% autologous plasma, 1% bovine serum albumin, 0.1% PHA (Wellcome, Dartford, England) 5% PHA-LCM, 10 μg/mL 12-0-tetradecanoylphorbol-13-acetate (TPA) (Sigma, St Louis), and 0.3% agar in Iscove's modified Dulbecco medium (IMDM). In some experiments, 10 U of recombinant interleukin 2 (Biogen, Geneva) were added. After seven days of culture at 37°C in a humidified atmosphere of 5% CO_2 in air, aggregates containing more than 40 cells were counted as colonies.

With this assay more than 95% of the colonies develop in close vicinity to the agar surface and can easily be harvested by thorough floating of the agar surface with phosphate-buffered saline (PBS).[21] Ninety-four percent of the cells derived from colonies grown from the patient's blood stained positive with the pan T-cell monoclonal antibody Leu-4 (Becton Dickinson, Mountain View, CA) but lacked surface markers known to be present on myeloid, monocytic, and B cells.

Cytogenetic studies. Direct preparations of bone marrow cells and of unseparated peripheral blood cells stimulated with PHA were stained with Giemsa, and R- and Q-banded as described previously.[22]

For chromosome analyses of the passaged stromal cells grown from LTC one of the following methods was used. If the adherent cell layer was thin with numerous gaps between individual cells, the chromosome preparation was performed in the culture dish by adding Colcemid (Demecolcin; CIBA, Wehr, FRG) (final concentration 0.06 μg/mL) directly to the long-term medium. After one hour, all of the medium was removed and the adherent cells were washed twice with 0.075 mol/L KCl, 4 mL of which were left in the dish for 20 minutes. The hypotonic solution was replaced with fixative (3:1 methanol:acetic acid) and finally removed. After two

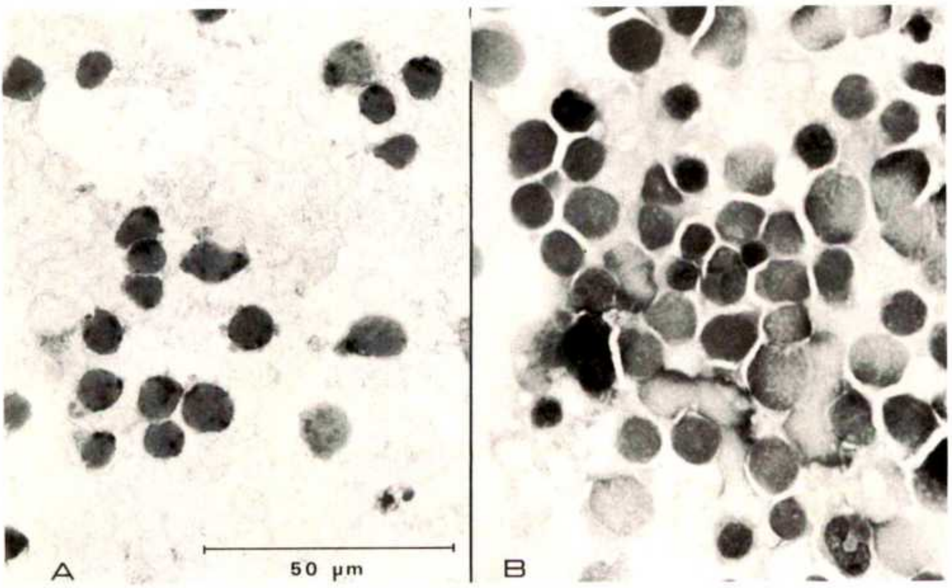

Fig 1. Acid phosphatase reaction of the patient's blast cells at diagnosis (A) and at the time of last relapse (day +1485 post BMT) (B).

washings with fixative the culture dishes, with the passaged stromal cells remaining in situ, were gently dried, followed by cytogenetic analysis.

If the cells forming the passaged stromal cell layer had grown to confluence before chromosomal analysis could be undertaken, Colcemid was added to the culture dish as described, but the layer was then rinsed with PBS, trypsinized,[17] and handled as described.[22] Suitable metaphases were G- or Q-banded according to published methods.

BFU-E were prepared by adding Colcemid (final concentration 0.045 μg/mL) to the culture dish for one hour at 37°C. Individual BFU-E were plucked, put into tubes containing 0.1 mL 0.075 mol/L KCl for resuspension, transferred onto polylysine–coated microscope slides[23] and kept in hypotonic solution for 35 minutes. Fixative was then dropped onto the area where the colony had been placed and the slide was air–dried and stained as described. Only single colonies in which two or more metaphases could be analyzed were accepted.

CFU-C and T-lymphocyte colonies were treated similarly except for chromosome analysis, which proved necessary to pool colonies, in order to obtain a sufficient number of mitoses.

HTLV-I antibodies. Sera were tested for the presence of antibodies against the human T-lymphotropic virus type I (HTLV-I) by enzyme-linked immunoadsorbent assay (ELISA)[24] and Western blot analysis. For the Western blot, HTLV-I was used as antigen that previously had been concentrated from cultured medium supernatant of MT-2 cells. The virus preparation was separated on SDS-PAGE and blotted to nitrocellulose. The blot was saturated with 1.5% BSA, 0.1% Ficoll, 0.1% polyvinylpyrrolidone and 0.05% gelatin (Cohen–buffer) for two hours at room temperature. Diluted sera (1:50 to 1:200 in PBS) were incubated overnight, washed, and then incubated with antihuman IgG (1:1000), conjugated to peroxidase, and reacted with diaminobenzidine.

RESULTS

Chromosome analyses performed on the patient's bone marrow and the donor's lymphocytes before BMT had shown normal female and male karyotypes, respectively. Thirteen and 605 days after BMT, cytogenetic studies on the patient's marrow or peripheral blood cells revealed a male pattern with no abnormalities.

Attempts to investigate the chromosomes at the time of first relapse after BMT (day +878) were unsuccessful because no mitoses were obtained either with or without the methotrexate synchronization technique. On day +1039, when a remission had been induced but reappearing clusters of immature lymphoid cells within an otherwise normal marrow suggested the resurgence of leukemia, 40 of 41 mitoses were male (46,XY); one metaphase lacked one of the sex chromosomes and one chromosome 7(44,X,−7).

The results of detailed cytogenetic analyses done at the time of third relapse (second relapse after BMT) (day +1485) and the following remission (day +1557) are summarized in Table 1.

At relapse, 17 of 20 marrow cells analyzed after direct preparation were male. In two of these, the long arm of chromosome 6 was partially missing, the karyotype being 46,XY, del(6) (q23q25) (Fig 2). A more detailed view of both chromosomes 6 demonstrating the interstitial deletion of one of the chromosomes is given in Fig 3. The same abnormality was also found in metaphases obtained from PHA-stimulated peripheral blood cells (two mitoses), T-cell colonies, and passaged adherent layer cells (one mitosis each). A second new clone characterized by a loss of one of the sex chromosomes in combination with several other abnormalities including a 6q− chromosome (45,X,−4, del(6)(q23q25),+8,−15,−21,+i(21q),+mar) was found in the remaining three metaphases obtained after direct marrow preparation. Because recipient and donor had no differing fluorescent polymorphisms, we were unable to decide if this clone was host- or donor–derived. The five mitoses found after stimulation of peripheral blood cells with PHA and the vast majority of karyotypes originating from T-cell colonies were male (66 of 69 mitoses), three mitoses were female. On the contrary, 17 of 19 metaphases from the passaged adherent cell layer were of female origin, two mitoses were male, with one of these bearing the 6q− anomaly.

After a remission had been achieved, exclusively normal male karyotypes were found in the myeloid and the lymphoid compartment. The marrow stroma showed fourteen host metaphases; two mitoses were of donor origin.

Both patient and donor lacked antibodies against HTLV-I, as judged by ELISA and Western blot analysis.

DISCUSSION

A deletion of the long arm of chromosome 6 was first described in 1976 as a specific structural anomaly found in the leukemic cells of patients with ALL.[25] This initial report was confirmed by the Third International Workshop on Chromosomes in Leukemia[12] where 6% of 216 cases of ALL carrying any chromosome abnormalities exhibited a 6q-chromosome. At least 25 cases have now been reported,[26-29] the majority of which seemed to belong to the non-T cell non-B cell subgroup of ALL with isolated cases being of T cell origin.

Table 1. Results of Chromosome Studies Performed at Second Relapse After BMT (Day +1485) and in Remission (Day +1557), Grouped According to the Origin of Cells Taken for Cytogenetic Analysis

	Direct Bone Marrow		PHA-stimulated Peripheral Blood	T-Cell Colonies		BFU-E	CFU-C	Passaged Adherent Layer From LTC	
Karyotype	Relapse	Remission	Relapse	Relapse	Remission	Remission	Remission	Relapse	Remission
46,XY	15	33	3	65	56	12	6	1	2
46,XY,6q−	2	0	2	1	0	0	0	1	0
45,X,6q−*	3	0	0	0	0	0	0	0	0
46,XX	0	0	0	3	0	0	0	17	14†

Abbreviation: LTC, long-term cultures.

*Full karyotype: 45,X,−4,del(6)(q23q25),+8,−15,−21,+i(21q),+mar.

†Eleven mitoses of female origin carried nonclonal abnormalities.

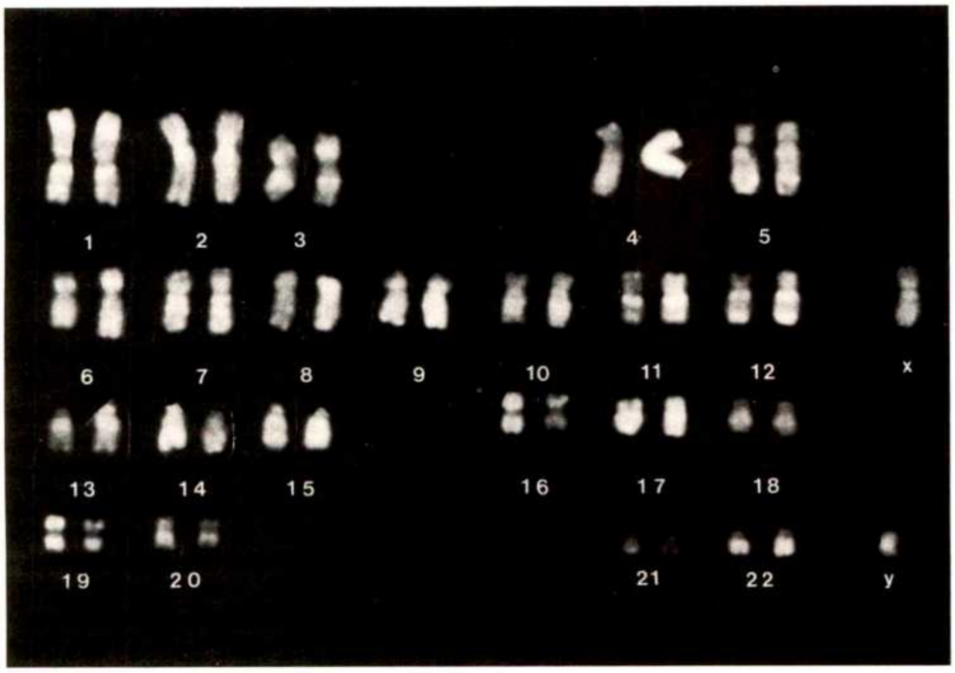

Fig 2. Metaphase of a directly prepared bone marrow cell at relapse (day +1485). Note the interstitial deletion of one chromosome 6 and the bright fluorescence of the Y chromosome.

In the present case the discovery of an interstitial deletion of the long arm of chromosome 6 in male metaphases adds substantial evidence that the malignant transformation had actually occurred in donor cells. As the mitotic activity of various hematopoietic cell lineages after direct preparation of marrow cells may vary greatly according to the culture conditions in use,[30] one cannot exclude the possibility that the demonstration of a normal karyotype in a leukemic marrow merely indicates that only members of the residual normal hematopoiesis have been analyzed while the leukemic cells went undetected because of their blocked mitotic activity. This could also have happened in cases where normal donor metaphases were found at relapse after BMT,[31] and the donor cells might then have represented the residual normal population originating from the marrow graft, while the leukemic clone—possibly of host origin—was missed. The detection of a disease-specific chromosome abnormality can avoid such ambiguities and gives more direct proof that donor cells were involved in the leukemic process.

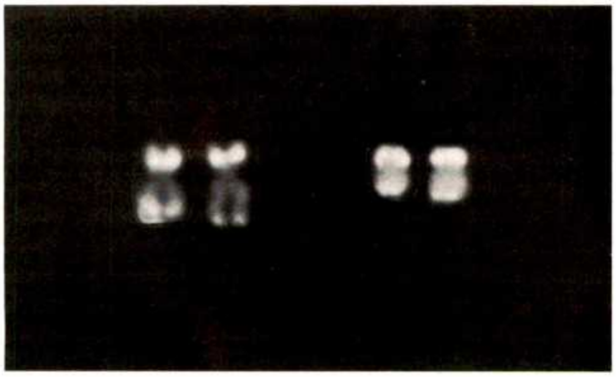

Fig 3. Two partial karyotypes showing the interstitial deletion of one chromosome 6 (del(6)(q23q25)) at different time points of the metaphase.

On the other hand the demonstration of the 6q− anomaly supports the diagnosis of ALL at the time of last relapse. The absence of the typical acid phosphatase reaction at that time, however, when compared with the unequivocal staining pattern of blast cells at diagnosis casts some doubt as to whether it really was the original leukemia that recurred after BMT. Indeed, for the only additional case of late donor cell leukemia reported so far, Witherspoon et al presented evidence that the leukemia occurring six years postgrafting arose from a cell that was different from that of the original leukemia.[8] The long latent period between transplantation and expression of the leukemic transformation after BMT would also support their view that other than the previously discussed mechanisms[2,3,6-8] might be responsible for the development of leukemia in donor cells late after allogeneic BMT. For example, it has been proposed that more than one oncogene may be required for the ultimate transformation.[32,33]

Regarding the mechanisms of relapse in donor cells after BMT, two aspects of our case deserve further comment. First, the oncogene c-myb, probably coding for a protein that is important in the early differentiation steps of hematopoietic cells, has been localized to the long arm of chromosome 6 within bands q22-24,[34] which is in the region of the breakpoints found in most cases of ALL associated with a 6q− chromosome. What particular implications, if any, this observation might have in our case is not clear. Second, adult T-cell leukemia, which is endemic in southwestern Japan but sporadically found in other parts of the world, seems correlated to abnormalities of chromosome 6. More than half of 30 Japanese patients carrying high titers of HTLV-I antibodies exhibited a 6q− chromosome.[35] As both leukemic clones found in our patient showed the 6q− anomaly and blast cells were T cell derived, at least before BMT, it seemed reasonable to test for antibodies against HTLV-I. Both recipient and donor were negative for HTLV-I antibodies, however, and thus we cannot explain the relapse in donor cells as a result of transmission of virus particles from recipient to donor tissue.

Little is known about the kinetics of relapse after BMT and in particular how different lymphohematopoietic lineages and the marrow stroma are involved. Using chromosomal studies of direct marrow preparations and mitogen-stimulated blood cultures, Lawler et al defined various chimeric states related to leukemic relapse after grafting.[31] The pattern they reported in patients with relapsed ALL was the coexistence of recipient and donor metaphases in the marrow while lymphoid cells from the peripheral blood remained of donor type. Our case fits this description in its lymphoid parts, while the fact that all dividing cells found after direct marrow preparation were of donor origin would appear a rare exception to the rule. Incomplete chimerism in cells from T-cell colonies obviously is not infrequent, as we observed this phenomenon in three of 12 patients early after BMT (manuscript in preparation).

The origin of the marrow stroma after BMT is still controversial. While several investigators have demonstrated that marrow fibroblasts remain host–derived,[36-38] Keating et al reported that cells from the adherent layer of long-term cultures became progressively donor in origin with increasing time after grafting.[39] This is in contrast to the findings in our patient (Table 1). The only explanation we have for this discrepancy is that Keating et al analyzed cells from the original adherent layer, while we used passaged stromal elements for cytogenetic studies. It is well known that the adherent layer of human LTC contains a substantial number of hematopoietic progenitors.[17] In line with these findings we were able to grow CFU-C (45 colonies per 10^5 mononuclear cells) from the patient's original adherent layer cells after culture of her remission marrow. Although colony–forming cells were no longer obtained from the adherent layer cells after a single passage, we believe that the few donor mitoses found after cytogenetic analysis of the first–passage stromal elements reflect the persistence of hematopoietic cells of the donor within a host–type marrow stroma rather than the existence of an incomplete chimerism within this compartment.

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Functional Heterogeneity of B-CLL Lymphocytes: Dissociated Responsiveness to Growth Factors and Distinct Requirements for a First Activation Signal

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We studied the effects of B cell directed growth factors on B lymphocytes from 11 patients with chronic lymphocytic leukemia (B-CLL). B-CLL lymphocytes were costimulated with anti-μ antibody (Ab) and with three growth factor preparations: recombinant IL2, B cell growth factor (BCGF) (20 kiloDalton (kD) BCGF) and a high molecular weight BCGF (50 kD BCGF). IL2 was the more active factor (in six of 11 patients). The effect of IL2 was dependent on a costimulation with anti-μ Ab or occurred independently of anti-μ Ab, according to the patients. This pattern of reactivity did not correlate with the presence or absence of the IL2 receptor (IL2-R) molecule on fresh B-CLL lymphocytes. Five patients responded to the 20 kD BCGF. Although four of them were also strong responders to IL2, one strongly responded to the 20 kD BCGF and did not respond to IL2. Only one patient responded to the 50 kD BCGF. When an anti-IL2-R Ab was introduced into the culture, only the responsiveness to IL2 was abolished: thus both 20 kD and 50 kD BCGFs activate B-CLL lymphocytes independently of the IL2-R. These results show that several B cell directed growth factors can act independently to support the proliferation of B-CLL lymphocytes.

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CHRONIC LYMPHOCYTIC leukemia of the B type (B-CLL) is a malignant disease characterized by the proliferation of monoclonal mature B lymphocytes. Several works showed that B-CLL lymphocytes can be induced to differentiate[1-3] or proliferate[4] in vitro on stimulation with mitogens or interleukins. It is thus important to determine the pattern of reactivity of B-CLL lymphocytes to normal B-cell directed biological mediators.

The early stages of B cell activation require two different kinds of signals: (1) the cross-binding of membrane IgM by the antigen, which can be mimicked by anti-μ antibody (Ab) or by *Staphylococcus aureus* Cowan 1[5]; and (2) T cells and/or T cell derived interleukins. Since the initial descriptions of B cell growth factors (BCGF) different from IL2[6,7] several BCGFs of human origin have been described.[8-12] In addition IL2 is able to promote directly the proliferation of activated normal and leukemic B cells.[13-17] Moreover, activated B cells have been shown to express receptors for IL2 indistinguishable from those of activated T cells.[18-21]

We examined the ability of B-CLL lymphocytes to respond to three different growth factors: recombinant IL2, and two BCGFs characterized by different molecular weights and by different functional properties. The latter will be referred to as 20 kD BCGF[10] and 50 kD BCGF.[11] We show that B-CLL lymphocytes from different patients vary in their responsiveness to these growth factors. Importantly, they may in some cases be directly triggered by these factors, in the absence of a first signal. In one such case, when the direct responsiveness to IL2 is considered the IL2 receptor (IL2-R) molecule was not present on fresh B-CLL cells. Rather, culture with IL2 itself was able to drive B-CLL lymphocytes to express the IL2-R molecule and to acquire the responsiveness to this interleukin.

MATERIALS AND METHODS

Patients. Eleven patients with CLL were studied. Their age range was between 60 and 80. All had stage A disease, characterized by the presence in peripheral blood of 20 to 50 × 10^3 leukocytes/mm^3, of which 60% to 90% were lymphocytes. They were not treated at the time of blood drawing, except patient ROU, whose lymphocytes were studied several times before and on treatment with identical results. No selection was operated on patients fulfilling these criteria. The hematological characteristics and surface markers of these patients' lymphocytes appear in Table 1.

Isolation of B-CLL lymphocytes. Mononuclear cells were isolated from B-CLL patients' blood samples by centrifugation on Ficoll-Hypaque. T cells were eliminated by two cycles of rosetting using aminoethyl isothio uronium bromide treated sheep RBCs. T depleted cells were then depleted in adherent cells by 45 minutes incubation on plastic petri dishes in the presence of 10% fetal calf serum (FCS). These preparations were analyzed using a FACSTAR flow cytometer. They were homogeneous small cells and contained less than 1% cells labeled by the OKT3 antibody (Ortho Pharmaceuticals, Raritan, NJ) and <1% cells labeled by the OKM5 antibody (Ortho Pharmaceuticals). These cells will be referred to as B-CLL lymphocytes.

Growth factors Recombinant IL2 was a gift of Biogen (Geneva). The preparation used had an estimated activity of 10^7 U/mg protein and contained 97.3% IL2 by sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) analysis.

BCGF purified by chromatography according to Mehta et al[10] was purchased from Cellular Products (Buffalo) This preparation did not contain detectable IL2 as assessed by the proliferation of mitogen activated human T cells and of the CTL-L murine T cell line. This growth factor will be referred to as 20 kD BCGF.

The 50 kD BCGF was prepared as already described.[11] Conditioned medium was produced by activating normal peripheral blood lymphocytes with phytohemagglutinin The supernatant was applied onto a concanavalin A sepharose column (Pharmacia Fine Chemicals, Uppsala, Sweden) and the active material eluted with α-D-methyl mannoside (Sigma, St Louis) in 0.15 mol/L NaCl. This preparation contained no IL2 and supported B cell proliferation. The material used in the present work was further purified by two different chromatographic methods[22]: gel filtration on an ACA 54 column (IBF, Villeneuve, France) (the active fraction had an

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Table 1. Selected Data From Patients With B-CLL

Patients	Age/Sex	Blood Leukocytes Count ($\times 10^{-3}$/mm^3)	sIg	Leukemic Immunophenotype (Percentage of Positive Cells)* B1	B4	HLA-DR	Leu 1	OKT11
BAT	81/F	22	$\mu\lambda$	50.4	83.2	86.2	76	27.3
LEG	66/M	19.8	$\mu\kappa$	69	79	76	80	10
LOI	76/M	38 4	$\mu\kappa$	63.5	93	89 8	74.6	16.2
RIA	65/M	22 3	$\mu\lambda$	80 3	72.8	89	84	27.5
POUB	78/F	54.2	$\mu\kappa$	53.2	84 8	83.7	77.8	15
ROU	74/M	26.1	†	57	76	76	78	12
PER	71/M	48.5	$\mu\kappa$	59	59.2	76	80	10
BRU	65/M	99	$\mu\kappa$	52.91	91	96	92	27
GUI	78/F	41	$\mu\kappa$	60.3	91.3	87.6	89.6	17
ALB	70/M	24.4	$\mu\lambda$	54	81	75.9	72	16.5
POU	75/F	32.5	$\mu\lambda$	51	72	76	65	13.5

*Peripheral blood lymphocytes from leukemic patients were phenotyped by indirect immunofluorescence assay using FITC conjugated goat anti-mouse IgF(ab)$'_2$. Surface membrane immunoglobulin (sIg) phenotype was assayed by immunoperoxidase.

†Staining too weak to permit identification

Abbreviations: μ, heavy chain; λ, light chain.

apparent MW of 50 kD) and affinity chromatography on a Biogel HPHT hydroxylatatite column (Bio-Rad, Richmond, CA) (the active fractions were eluted at phosphate concentrations between 160 and 180 mmol/L). This material, referred to as 50 kD BCGF, contained no IL2, IL1, or antiviral activities.

B cell proliferation assay. In costimulation assays insolubilized anti-μ Ab (Bio-Rad) was used at a final concentration of 10 μg/mL. This concentration was selected on the basis of preliminary experiments using normal B cells or B-CLL lymphocytes. B-CLL lymphocytes were cultured in 96 well flat-bottomed microtiter plates at a concentration of 10^5 cells per well in 0.2 mL culture medium. The latter was RPMI 1640 supplemented with 10% FCS and 5×10^{-5} mol/L 2-mercaptoethanol. The cultures received graded concentrations of IL2, or serial dilutions of BCGFs in the presence or in the absence of anti-μ Ab. After three and six days of culture the proliferative response was assessed by the addition of 0.5 μCi (^{3}H)thymidine (CEA, France) during the last 16 hours of culture. Cultures were performed in duplicate and the standard deviation between replicates was <5%. When the day 3 and day 6 responses were considered, the day 6 response was identical to or higher than the day 3 response Thus only the latter response was considered. A patient's lymphocytes were considered as responsive to a given factor when this factor induced a dose-related response, with a maximal thymidine incorporation at least five times that observed in the presence of anti-μ Ab and in the absence of the factor.

Functional effect of anti–IL2-R antibodies. In one case an anti–IL2-R Ab was used to inhibit the IL2 dependent proliferation of B-CLL lymphocytes. In this case we used the 33.B.3-1 Ab produced by Olive et al. This Ab is a rat IgG$_{2a}$ directed toward the IL2-R molecule and able to inhibit the IL2 dependent proliferation of normal T[23] and B cells.[22] The blocking experiments were performed as follows: B-CLL lymphocytes were cultured for three days at 2×10^6 cells per milliliter in the presence of anti-μ Ab (10 μg/mL). They were then washed extensively, cultured for three additional days in the presence of graded concentrations of IL2 or 20 kD BCGF or 50 kD BCGF and in the absence or in the presence of the 33-B-3.1 Ab. The 33-B-3.1 Ab obtained as ascitic fluid was used at a final dilution of $^1/_{1000}$ and added to the cultures two hours before the addition of IL2.

Analysis of activation markers on patient's lymphocytes before and after three days of culture. For determination of the presence of activation markers on B-CLL lymphocytes we used the following antibodies: OKT9,[24] an Ab toward the transferrin receptor; anti-Tac Ab (a gift of T. Waldmann), directed toward a molecule associated with IL2-R[25], 4F2 (a gift of A. Fauci), an early activation marker for B lymphocytes[26]; and the 1.35 anti-DR Ab,[27] to quantify the expression of MHC class II products. Selected patients' lymphocytes were cultured for three days at 2.10^6 cells per milliliter with medium, with anti-μ Ab (10 μg/mL) or with IL2 (4 U/mL) according to the functional data obtained in previous experiments. Cells were washed and 0.7×10^6 viable cells were treated with antibodies at appropriate dilution in phosphate buffer saline (PBS) containing 2% bovine serum albumin (BSA) and 0.1% sodium azide (PBS/BSA/Azide). After 30 minutes incubation at 4°C, the cells were washed twice in cold PBS/BSA/Azide, resuspended in 50 μL of $^1/_{10}$ dilution of FITC-conjugated goat F(ab)$'_2$ anti-mouse Ig (Tago, Burlingame, CA) and incubated for 30 minutes at 4°C. Thereafter, cells were washed twice in PBS/BSA/Azide. Fluorescence analyses were performed on a FACSTAR flow cytometer.

RESULTS

Response of B-CLL lymphocytes to IL2. B-CLL lymphocytes were cultured in the presence of (4 U/mL) of IL2 and in the presence or absence of anti-μ Ab (10 μg/mL). Both reagents were added on day 0 of the culture. In the absence of growth factor, anti-μ Ab had no effect (Table 2). In five cases IL2 did not induce thymidine incorporation in B-CLL lymphocytes, regardless of the presence of anti-μ Ab (POUB, BRU, GUI, ALB, and POU). The other six patients' lymphocytes were able to proliferate in the presence of IL2 and this response was dose-related. In five cases (BAT, LEG, LOI, RIA, and PER) a clearcut costimulatory effect of IL2 and anti-μ Ab was apparent. In one case (ROU) an optimal response to IL2 was obtained in the absence of anti-μ Ab, the addition of which had no additive or synergistic effect.

Response of B-CLL lymphocytes to 20 kD BCGF. The proliferative response to the 20 kD BCGF was tested in the same experiments. Comparable experimental conditions were used: concentrations of 20 vol/vol dilution of 20 kD BCGF were added to the cultures in the presence and absence of anti-μ Ab. Lymphocytes from five of 11 patients responded to the 20 kD BCGF. The pattern of reactivity of these five patients' lymphocytes to the 20 kD BCGF was heterogeneous. In one case this response was not consistently

Table 2. Effect of IL2, 20 kD BCGF, and 50 kD BCGF on B-CLL Proliferation

Patients	(^{3}H) Thymidine Incorporation*							
	Without Anti-μ Ab				With Anti-μ Ab			
	—	IL2	20 kD BCGF	50 kD BCGF	—	IL2	20 kD BCGF	50 kD BCGF
BAT	761	7,800	6,178	2,641	980	42,200	9,496	2,896
LEG	943	8,156	7,898	3,566	868	41,398	12,698	4,202
LOI	307	1,798	567	368	786	15,941	2,156	608
RIA	259	1,645	15,404	33,380	259	13,872	10,396	30,111
POUB	1,714	3,051	11,607	ND	896	1,594	9,343	ND
ROU	1,245	53,627	2,458	1,435	1,381	56,527	1,987	1,396
PER	382	1,219	5,327	ND	1,830	17,176	11,756	ND
BRU	299	1,050	1,975	801	540	1,099	1,783	1,606
GUI	277	500	312	492	536	1,750	2,010	514
ALB	402	1,500	1,221	369	378	2,500	1,351	363
POU	370	1,336	2,567	ND	550	675	2,002	ND

Proliferation was measured by incorporation of ^{3}H thymidine during 16 hours pulse Data shown represent the mean of duplicate cultures

Abbreviation: ND, not done.

*10^5 purified B-CLL lymphocytes were cultured with IL2 (4 U/mL), with 20 kD BCGF (20% vol/vol), or with 50 kD BCGF (20% vol/vol), in the absence or in the presence of anti-μ Ab (10 μg/mL) for six days. Standard deviation was <5%

influenced by the presence of anti-μ Ab (BAT). In two cases a clearcut costimulatory effect of anti-μ Ab and 20 kD BCGF was observed (LEG and PER). In two cases (RIA and POUB) the 20 kD BCGF stimulated thymidine incorporation in the absence of anti-μ Ab, and this latter reagent did not enhance the response.

The lymphocytes from four patients responding to the 20 kD BCGF exhibited a clearcut response to IL2 (BAT, LEG, RIA, and PER). Interestingly, the lymphocytes from one patient displayed a clearcut response to 20 kD BCGF, in contrast to no response to IL2 (POUB). The same discrepancy was observed when this patient's lymphocyte response was measured on day 3 (data not shown).

Response of B-CLL lymphocytes to the 50 kD BCGF. The responsiveness of B-CLL lymphocytes to the 50 kD BCGF in the absence and presence of anti-μ Ab was tested in eight patients. RIA's lymphocytes were able to respond to the 50 kD BCGF. This response was obtained regardless of the presence of anti-μ Ab. Lymphocytes from this patient also responded to both 20 kD BCGF and IL2. However, the response to the former factor was anti-μ independent, whereas that to IL2 required costimulation with anti-μ Ab.

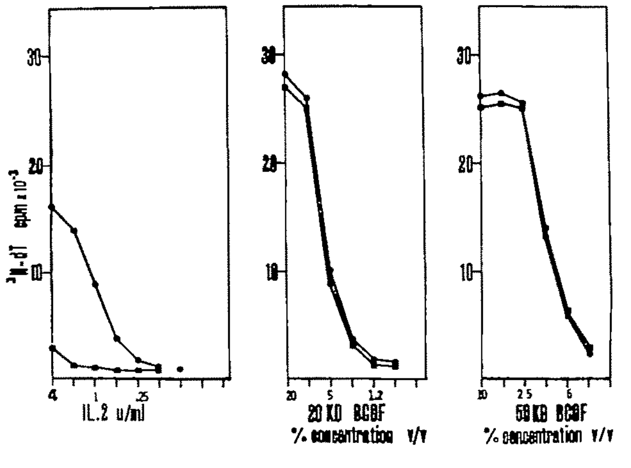

Fig 1. RIA's lymphocytes were cultured at 2 × 10^6 cells per milliliter with anti-μ Ab (10 μg/mL) for three days. Cells were washed extensively and cultured at a concentration of 10^5 cells per well for three additional days with graded concentration of Interleukins in the absence (●—●) or in the presence (■—■) of 33B.3.1 (anti-IL2-R Ab). The proliferative repsonse was evaluated by (^{3}H) thymidine incorporation.

Involvement of IL2-R in the response of B-CLL lymphocytes. We wished to verify that the responsiveness to the BCGFs occurred independently of the IL2-R molecule. Lymphocytes from patient RIA, who responded to all three growth factors, were selected. They were cultured for three days in the presence of anti-μ Ab, washed and recultured for three additional days in the presence of any of the three growth factors (Fig 1). The addition of an antibody directed toward a functional epitope of the IL2-R molecule fully inhibited the response of IL2, without affecting the dose effect curves of BCGFs. Thus the 20 kD and 50 kD BCGFs interact with B-CLL lymphocytes independently of the IL2-R molecule.

Acquisition of activation markers by B-CLL patients' lymphocytes. We examined the presence of activation markers on the lymphocytes from three patients selected as representative of a functional pattern of responsiveness. When their lymphocytes were examined before culture, they did not express Tac, 4F2 or T9 antigens (Table 3). The cells from these patients were cultured for three days in conditions previously shown to induce or not induce B cell proliferation. The phenotypic analysis was then performed (Table 4). Patient RIA's lymphocytes were cultured with anti-μ Ab, a procedure that allows the response of these cells to IL2.

Table 3. Lack of Activation Markers Expression Before Culture

Patients	Mean of Fluorescence Intensity			
	HLA-DR	Tac	4F2	OKT9
RIA	46.07	7.21	11.2	8 5
BRU	58.53	11.79	9.3	9.25
ROU	63.63	9.17	8 88	6.52

B-CLL lymphocytes from three selected patients were analyzed for the expression of activation markers before culture. Fluorescence was determined as described in Materials and Methods

Table 4. Surface Membrane Antigen on Activated B-CLL

Patients	Inducers	Mean of Fluorescence Intensity HLA-DR	Tac	4F2	OKT9
RIA	Medium	49	8 70	15.28	12.13
	Anti-μ Ab	80	24.42	22 46	13.82
BRU	Medium	76.77	13.43	9 02	5.54
	Anti-μ Ab	110.35	13 21	7.37	5 68
ROU	Medium	146.78	15.63	11.45	8.82
Experiment 1	IL2	348.25	35.51	21.60	16.88
Experiment 2	Medium	10 75	1.91	N.D.	
	Anti-μ Ab	8.35	1 54		

2 × 10^6/mL B-CLL lymphocytes from three selected patients were cultured with medium, with anti-μ Ab (10 μg/mL), or with IL2 (4 U/mL) for three days

RIA's lymphocytes expressed the Tac, 4F2 antigens and an increased density of class II products upon culture with anti-μ Ab, but not when cultured alone. Patient BRU's lymphocytes were unreactive to all three growth factors, even in the presence of anti-μ Ab. In this case anti-μ Ab did not modify the density of Tac antigen. Patient ROU's lymphocytes were directly reactive to IL2. On culture with this factor (but not without additon or with anti-μ Ab) we observed the correlated increased expression of all activation markers tested: class II, Tac, 4F2, and T9 antigens (Fig 2).

DISCUSSION

The initial steps of B cell activation are usually thought to result from the combined effect of the antigenic signal and of T cell derived interleukins.[4,5] Using anti-Ig antibodies to mimic the antigenic signal, several studies have characterized B cell directed growth factors, able to drive normal B cells into the S phase of the cell cycle. In the human at least three different growth factors can act in synergy with anti-μ Ab: (1) a 20 kD MW BCGF distinct from IL2[6] that has been recently purified to homogeneity[10]; (2) IL2-R or purified IL2 from various sources[13-15]; (3) higher MW BCGF(s) produced by mitogen activated PBL,[9,11] by lymphoblastoid cell lines,[28] or by T cell clones.[12] The precise relationships between these latter factors are not yet fully defined.

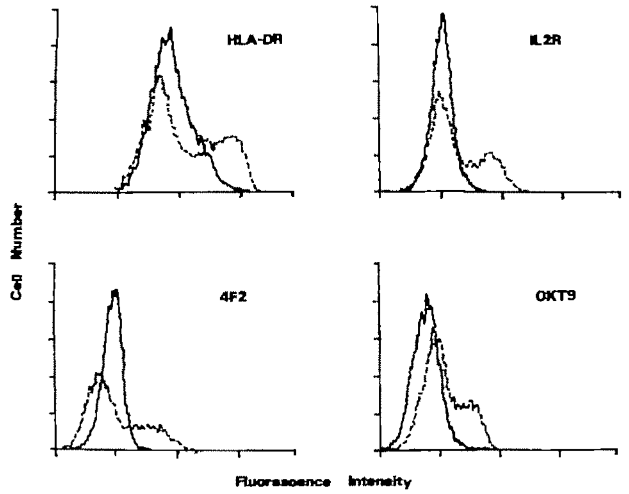

Fig 2. ROU's lymphocytes were cultured with medium (——) or with IL2 (----) for three days then washed and stained as described in Materials and Methods.

In this work, using costimulation with anti-μ Ab, we studied the reactivity of B-CLL lymphocytes to such growth factors, the 20 kD BCGF, IL2, and a preparation containing a 50 kD MW BCGF. Four of 11 patients' lymphocytes were unreactive to all three growth factors. This lack of responsiveness may not be absolute as anti-μ Ab, used as a first signal in our study, may not be the optimal stimulus for B-CLL cells, which have a low membrane Ig density.[29,30] In six cases patients' lymphocytes responded to IL2, which is in line with results previously reported by others.[16] Five of 11 patients' lymphocytes responded to the 20 kD BCGF. Among those, four were also strong responders to IL2. It should be pointed out that in a fifth patient a clearcut response to the 20 kD BCGF contrasted with a nonresponse to IL2. One individual patient's lymphocytes responded to IL2, to 20 kD BCGF, and to 50 kD BCGF.

The combined effect of a first signal and of T cell derived factors is usually considered as a requirement for the triggering of the activation of resting B cells and their progression into the cell cycle.[5] The cells from our patients were small sized (putatively resting) B lymphocytes. We verified in three selected cases that they did not express the activation markers Tac, 4F2, and T9 before culture. This was especially important in these three cases where B-CLL lymphocytes responded to growth factors in the absence of anti-μ Ab. These results emphasize the functional heterogeneity of small B cells.[31]

In another series of experiments we analyzed the conditions for the in vitro acquisition of the IL2-R molecule and its relationships with that of activation markers. When patient RIA's lymphocytes were cultured for three days with anti-μ Ab (a procedure able to induce their responsiveness to IL2) they acquired the Tac antigen and increased their expression of 4F2, and major histocompatibility complex (MHC) class II products. In contrast, cells from the unresponsive patient BRU were not modified by the same incubation procedure.

The direct responsiveness to a given growth factor could be expected to correlate with the constitutive expression of the corresponding receptor. We examined this point for IL2 responsiveness using the IL2 responsive cells from patient ROU. These cells were Tac$^-$ before culture. A three day culture in the absence of reagent or in the presence of anti-μ Ab (which did not influence the responsiveness to IL2) did not induce Tac positivity. In contrast, these cells became Tac$^+$ (and remained negative for the T_3 marker) after a three-day culture in the presence of IL2. The presence of Tac$^+$ cells on day 3 correlated with their responsiveness to IL2 on day 6. Our results confirm that IL2 can act positively on the expression of the IL2-R molecule[32] and show that this expression may be functionally significant. Moreover, on culture with IL2, patient ROU's lymphocytes expressed the 4F2 and the T9 antigens and increased their expression of MHC class II molecules.

B-CLL lymphocytes are thought to be frozen at various maturation stages[30] and these monoclonal populations may be representative of at least some aspects of the heterogeneity of normal B cells. It is therefore not surprising that the reactivity to the three growth factors studied varied among our patients' lymphocytes. From our results IL2 is the most frequently active growth factor. However, in five cases we observed a response to the 20 kD BCGF. This is in contrast to results of a recent report, showing the lack of reactivity of B-CLL lymphocytes to the same preparation of BCGF.[33] This discrepancy could be due to technical conditions or to patient selection. In our hands four of the five patients responding to 20 kD BCGF (among whom one responded to 50 kD BCGF) also responded to IL2. However, in one case (POUB), the response to IL2 was absent and contrasted with a clearcut response to the 20 kD BCGF. This latter result and the absence of IL2 in the BCGF preparations used clearly show that B-CLL lymphocytes may be responsive to BCGFs. On the other hand 20 kD BCGF has been shown to induce the proliferation of cells from another lymphoproliferative disease, namely hairy cell leukemia.[34,35] Moreover, the additional study of one patient (RIA) shows that the reactivities to IL2 and to BCGFs are indeed representative of different B cell activation pathways. Patient RIA's lymphocytes responded to BCGFs in the absence of anti-μ Ab, whereas they required anti-μ Ab to proliferate in the presence of IL2. More importantly a monoclonal Ab to the IL2-R molecule abolished the responsiveness to IL2 without affecting the dose-effect curves of BCGFs. The direct discrimination between the responsiveness to the 20 kD and to the 50 kD BCGFs could not be provided in this work. These growth factors differ with respect to their biochemical properties and several functional differences. Normal PBL large B cells (putatively preactivated in vivo) when cultured in the absence of anti-μ Ab do respond to the 20 kD BCGF, whereas they do not respond to the 50 kD BCGF.[11] However, PBL B cells may be directly responsive to the 50 kD BCGF in some auto-immune situations[36]: such B cells may be a normal counterpart of patient RIA's B-CLL cells. More importantly, the 50 kD BCGF does not support the 20 kD BCGF-dependent growth of a normal long-term B cell line[37] (A. Vazquez, unpublished results, 1985). Our results do not exclude that a given B cell subpopulation may be selectively responsive to a given growth factor. However, they show that monoclonal B cells (and thus at least some individual normal B cells) can respond simultaneously to several growth factors.

As exemplified by recent results obtained with hairy cell leukemia lymphocytes,[34,35] the analysis of the reactivity of lymphocytes in monoclonal lymphoproliferative disorders to growth factors may have important implications for the understanding and management of these diseases.

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The Molecular Species Composition of Phosphatidylcholine Affects Cellular Properties in Normal and Sickle Erythrocytes

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The phosphatidylcholine specific transfer protein (PCTP) from bovine liver was used to retailor the molecular species composition of phosphatidylcholine (PC) in the membrane of normal (AA) and sickleable (SS) human erythrocytes. Changes in molecular species composition of PC altered morphology as well as cellular deformability and stability as measured with ektacytometry. In normal cells, replacement of native PC with 1-palmitoyl,2-arachidonoyl PC (PAPC) resulted in a decrease in osmotic fragility with no change in hydration, whereas replacement with 1,2-dipalmitoyl PC (DPPC) led to an increased osmotic fragility and cellular hydration. Replacement of native PC by 1-palmitoyl,2-oleoyl PC (POPC) in normal cells had no apparent effect on these parameters. In contrast, replacement of native PC in sickle cells with either PAPC, DPPC or POPC led to cellular hydration. Facilitation of PC exchange between subpopulations of SS cells separated on buoyant density also led to cellular hydration. These observations suggest that the state of hydration of sickle cells can be modified by the fatty acyl composition of PC and illustrate a role for the lipid core in the observed permeability changes in sickle erythrocytes. They also raise the interesting possibility that the state of cellular hydration of sickle cells may be modulated by altering the molecular species composition of the membrane phospholipids.

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FATTY ACYL GROUPS and cholesterol are the major constituents of the hydrophobic interior of all biological membranes. This core provides a permeability barrier to hydrophilic substances at the boundaries of cells and cell organelles and serves as a matrix in which integral membrane proteins are embedded.[1] Each class of phospholipid in any particular biological membrane has a characteristic fatty acyl group composition. Increasing effort has been spent in recent years to understand the rationale for such a specific molecular species composition. Changes in fatty acyl unsaturation and chain length, with the accompanying change in the molecular structure of the phospholipid molecules, will affect membrane properties as shown in several studies of model membrane systems as well as biological membranes. The modification of fatty acids in the phospholipids of the membrane provides an approach to these questions, since the correlation of alteration in fatty acyl group composition and membrane properties can be measured.

Recently, the phosphatidylcholine specific transfer protein (PCTP) isolated from bovine liver was used to replace native phosphatidylcholine (PC) from intact erythrocytes with a variety of well-defined PC species.[2-4] This was accomplished by incubating erythrocytes in the presence of PCTP with vesicles of a well-defined composition. This treatment did not change the total phospholipid or cholesterol content of the membrane and demonstrated that the fatty acyl composition of PC in the human erythrocyte membrane can only be varied within limits without drastic effects on membrane structure and function.[2,5] For instance, it was shown that partial replacement of native PC by 1-palmitoyl,2-oleoyl PC (POPC), one of the major molecular constituents (30 to 40 mol/%) of human erythrocyte PC, had no apparent effect on the normal human red cell. However, replacement of native PC by 1,2-dipalmitoyl PC (DPPC) and 1-palmitoyl,2-arachidonoyl PC (PAPC), two minor constituents of human erythrocyte PC (5 to 10 mol/%), led to drastic changes in cellular functions. Replacement of native PC by DPPC resulted in echinocytic transformation, an increase in osmotic fragility, and hemolysis under isotonic conditions. Replacement of native PC by PAPC led to echinocytic shape change, increase in potassium permeability, and decrease in osmotic fragility.

In order to further define the cellular consequences of alterations in PC species composition of the red cell membrane we used ektacytometry to measure the deformability characteristics of normal and sickle cells following fatty acyl modification of PC with the three molecular species mentioned above. We found that cellular deformability of both normal and sickle erythrocytes was affected by the PC species composition of the membrane, primarily as a result of alteration in the state of cell hydration following the fatty acyl modification of PC. Although the primary defect of sickle erythrocytes resides within the hemoglobin,[6] abnormalities in the phospholipid bilayer occur.[7-10] These membrane abnormalities may play an important role in the pathophysiology of sickle cell disease. The findings reported in this study suggest a role for the lipid core in the permeability defect observed in sickle erythrocytes,[11] and raise the possibility that the state of cellular hydration of these cells can be modulated by altering the molecular species composition of membrane phospholipids.

MATERIALS AND METHODS

1,2-Dipalmitoyl-sn-glycero-3-phosphocholine, 1-palmitoyl,2-oleoyl-sn-glycero-3-phosphocholine, egg phosphatidate, cholesterol,

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and polyvinylpyrrolidone (PVP) (MW 360,000), were purchased from Sigma (St Louis). 1-Palmitoyl,2-arachidonoyl-sn-glycerol-3-phosphocholine was obtained from Avanti Polar Lipids, Inc (Birmingham, AL). 1-palmitoyl,2[^{14}C]oleoyl-sn-glycero-3-phosphocholine, and 1-palmitoyl, 2-[^{14}C]palmitoyl-sn-glycero-3-phosphocholine were purchased from New England Nuclear (Boston). [^{3}H]Glycerol trioleate was obtained from Amersham (Cardiff, UK). ^{51}Cr was purchased as sodium chromate in saline pH 8.0 from New England Nuclear.

Preparation of vesicles. Vesicles were prepared from a lipid mixture containing one of the three phosphatidylcholine species mixed with an equimolar amount of cholesterol, 6 mol% of egg phosphatidate and trace amounts of [^{14}C]-phosphatidylcholine and [^{3}H]glycerol trioleate, the latter serving as nonexchangeable marker. The lipid mixture was dried from a chloroform/methanol solution (2:1, vol/vol) and vesicles were prepared as described before[3] in a buffer containing 150 mmol/L NaCl, 25 mmol/L glucose, 1 mmol/L ethylenediaminotetraacetate (EDTA), 100 IU/mL penicillin, 100 μg/mL streptomycin and 10 mmol/L tris (hydroxymethyl) aminomethane (TRIS), pH 7.4 (referred to as incubation buffer throughout).

Erythrocytes. After obtaining informed consent, human venous blood was collected in heparinized tubes for normal (AA) individuals and homozygous sickle cell (SS) patients. The erythrocytes were pelleted by centrifugation for five minutes at 2,500 x g and washed three times with a tenfold excess of incubation buffer.The buffy coat was carefully removed by aspiration after each wash. While most experiments were carried out with unfractionated populations of erythrocytes, some experiments used erythrocytes with well-defined cell densities obtained using discontinuous stractan gradients [12]

Preparation of the transfer protein. The phosphatidylcholine specific transfer protein was purified from bovine liver according to Kamp et al and had a specific activity of five μmol PC exchanged per milligram protein per minute.[13] It was homogeneous on SDS-disc gel electrophoresis and was stored at a concentration of 75 μg/mL in 50% glycerol at −20°C. Before use, glycerol was removed by overnight dialysis at 4°C against 600 vol of incubation buffer. After dialysis, the volume of the protein solution was reduced by concentrating against polyethylene glycol (Aquacide III, Calbiochem, San Diego), until a protein concentration of 150 μg/mL was reached

Replacement of erythrocyte PC by different PC species from donor vesicles. Cells were preincubated at 37°C for one hour with gentle shaking under room air or nitrogen. The replacement reaction was started by the addition of a mixture of the PC vesicles and PCTP. The incubation of the resulting 30% cell suspension, containing a final concentration of 0.6 to 0.9 mmol/L vesicle PC and 3 μmol/L transfer protein, was continued under either air or nitrogen.

Aliquots were taken at timed intervals and added to 5 mL of incubation buffer at 37°C. Cells were isolated by centrifugation for five minutes at 2,500 x g and the residual vesicles and transfer protein were removed by two additional washes with a 50-fold volume of incubation buffer. Lipids were extracted from the erythrocytes according to the procedure of Rose and Oklander.[14] The extent of PC replacement was calculated from the specific radioactivity of the phosphatidylcholine fraction as described before.[2] A correction was made for the extent of contamination of erythrocytes with vesicles by measuring the amount of nonexchangeable marker [^{3}H]-glycerol trioleate present in the extracted lipids. ^{14}C POPC was used as a marker for the transfer of PAPC for practical reasons. Radioactively labeled PAPC was not readily available and POPC satisfactorily reflects the actual exchange process of PAPC from vesicles to red cell membranes.[15]

PC exchange between subpopulations of sickle cells. Subpopulations of sickle cells with a defined range of cell densities were

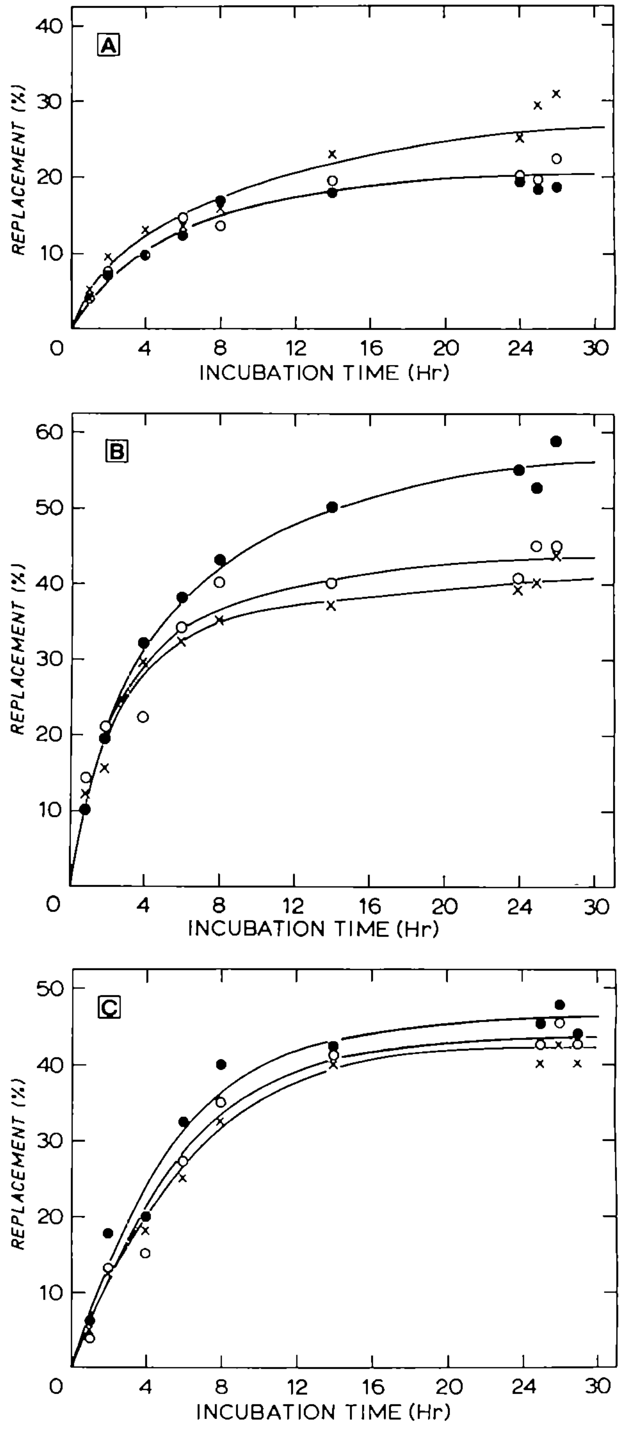

Fig 1. Replacement of native erythrocyte PC in normal RBC (x) and oxygenated (O) or deoxygenated (●) sickle RBC. (A) Replacement of native PC by DPPC, (B) replacement of native PC by POPC, and (C) replacement of native PC by PAPC. The extent of replacement was determined by using trace amounts of [^{14}C]dipalmitoyl PC (A) or 1-palmitoyl, 2-[^{14}C]oleoyl PC (B and C), respectively, in the donor vesicles. The resulting specific radioactivity of the erythrocyte PC was analyzed and data are expressed as percentage of total erythrocyte PC replaced by donor vesicle PC. During the exchange incubations, cells were kept under room air (x, O) or nitrogen (●). Oxygenated cells were preincubated at 37°C for one hour under room air; deoxygenated cells were preincubated at 37°C for one hour under humidified nitrogen.

harvested from a discontinuous stractan gradient and washed three times with 10 vol of incubation buffer. Erythrocytes with cell densities in the range of 1.0 to 1.0880 g/mL were incubated for two hours at 37°C under gentle shaking with vesicles containing egg PC and (^{14}C)1-palmitoyl,2-oleoyl PC, prepared as described above, in the presence of 2 μmol/L PCTP. The ratio of vesicle PC to erythrocyte PC was one to one. This incubation resulted in a partial replacement of erythrocyte PC by vesicle PC. The cells were subsequently separated from the vesicles and exchange protein as described above. These cells, now labeled with ^{14}C in the PC fraction, were incubated for one hour at 37°C at a hematocrit of 40% in the presence of 0.5 μCi^{51}Cr, and subsequently washed twice with buffer. Samples were taken to determine the specific radioactivity of ^{51}Cr per gram of hemoglobin and ^{14}C per gram of lipid phosphorus.

Aliquots of this double radio–labeled subpopulation were mixed with aliquots of the unlabeled high density subpopulations (cell density $>$1.0880 mg/mL) from the stractan gradient to reconstitute the original density distribution of the whole sickle cell population. This mixture of cells was incubated at 37°C overnight (14 hours) in presence or absence of PCTP. After this incubation cells were separated on a stractan gradient and the specific radioactivity of ^{51}Cr and ^{14}C was determined in each subpopulation.

Osmotic gradient ektacytometry. A Technicon ektacytometer was used to measure whole cell deformability as a continuous function of the suspending medium osmolality at a constant applied shear stress of 170 dynes/cm^2.[16] For these studies, the deformability index (DI) of erythrocytes was continuously recorded as the suspending medium osmolality was linearly increased from 50 to 500 mosm/kg. As previously shown, the curve relating the variation of DI with suspending medium osmolality can be analyzed to provide information about initial cell surface area, surface area–to–volume ratio, and cell water content.[16]

Cell morphology. Samples of 10 μL of washed packed cells were fixed for one hour at 20°C in 1 mL of buffer containing 100 mmol/L NaCl, 40 mmol/L Na citrate, and 0.5 mmol/L formaldehyde. After washing, the cells were post–fixed with 1% OsO_4 during 30 minutes and subsequently dehydrated in a graded series of ethanol, transferred into isopropanol, and the air dried. Cells were covered with a thin layer of gold by the sputter process. Microscopy was performed with a Cambridge Steroscan 600 M electron microscope.

RESULTS

Incubation of normal (AA) and sickle (SS) erythrocytes at 37°C in the presence of donor vesicles and the PC-specific transfer protein resulted in replacement of the native PC species of the erythrocyte membrane by the PC species from the donor vesicles (Fig. 1). Despite similar incubation conditions, a clear difference was seen in the PC replacement profiles for different PC species. The disaturated PC molecules (Fig 1A) were incorporated at a slower rate and to a lower extent than 1-saturated,-2-unsaturated species in both sickle and normal erythrocyte membranes (Fig 1B, C). The time course of replacement of PC species in sickle cells was very similar to that observed in normal erythrocytes except for the replacement reaction of POPC (Fig 1B). In this case, native PC of deoxygenated sickle cells was replaced by this particular species to a greater extent than in oxygenated sickle cells, indicating an increased pool of exchangeable PC

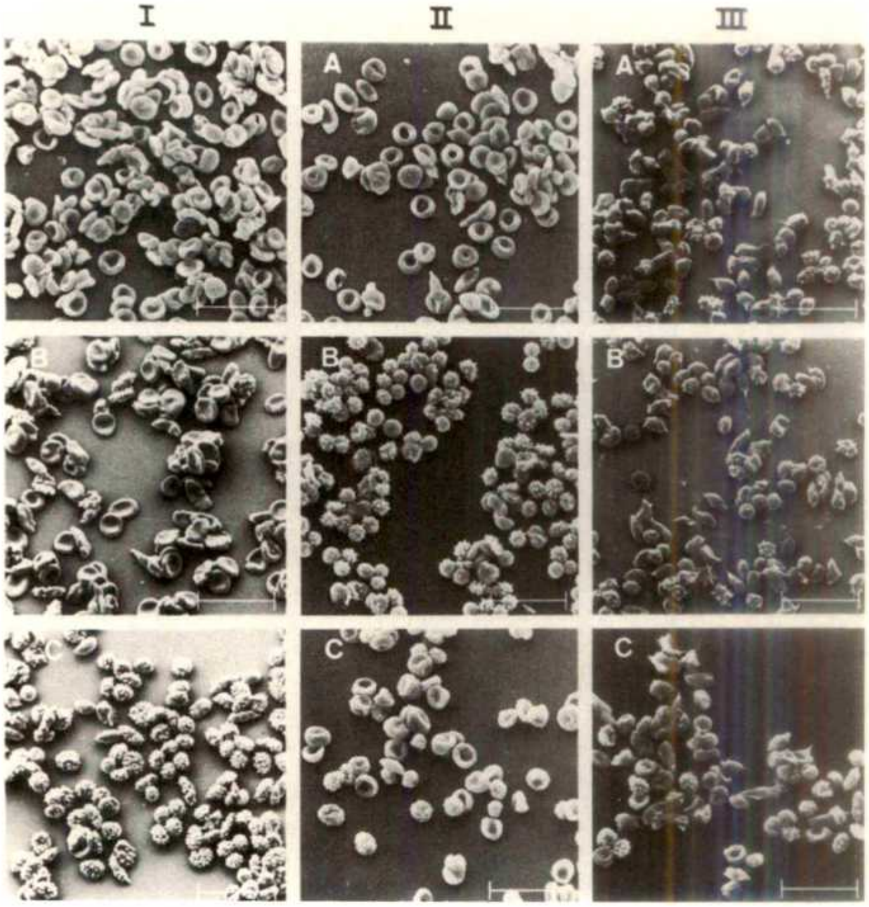

Fig 2. Electromicrographs of sickle red cells after partial replacement of their native PC. (I) Cells after four hours of incubation during which their native PC was partly replaced by POPC (A), DPPC (B), or PAPC (C) to an extent of 30%, 15%, or 20%, respectively. (II) Cells after 16 hours of incubation during which their native PC was partly replaced by POPC (A), DPPC (B) or PAPC (C) to an extent of 45%, 25%, and 40%, respectively. (III) Cells as depicted in II, after incubation under nitrogen for one hour. After reoxygenation, cells readopted their shape as shown in II.

in sickle cells under deoxygenated conditions. Deoxygenation had no effect on the replacement reaction of normal cells (data not shown). Figure 1 documents the exchange process as well as the level of replacement for each molecular species of PC. Using this measurement, we are able to relate the level of replacement with the observed effects on other cellular parameters.

Cell–shape. Replacement of the native PC in normal human RBCs by either DPPC or PAPC led to dramatic changes in morphology, whereas replacement of the native PC by POPC had no effect on the shape of the cell. Similar changes in morphology were observed in sickle cells under oxygenated conditions. Figure 2 shows that even when 45% of native PC was replaced by POPC, morphology was unchanged (Fig 2, IIA), while replacement of native PC with either DPPC or PAPC resulted in echinocytic tranformation. Replacement of only 15% of the native PC with DPPC led to the generation of a fair number of echinocytes (Fig 2, IB), while replacement of 25% of native PC with this molecular species resulted in almost complete conversion to the echinocytic shape (Fig 2, IIB). The most marked shape change was observed during replacement of native PC by PAPC. Replacement of 20% of native PC with this species led to a complete conversion into a crenated morphology with dimples (Fig 2, IC). After eight hours of PC exchange incubation, the spikes began to disappear, although the dimples were still present (Fig 2, IIC, and Fig 3).

Reversible sickle cells (RSC) in which the native PC had been replaced by either of the above mentioned species sickled following incubation under nitrogen (Fig 2, III). Cells in which part of the PC had been replaced by either DPPC or PAPC almost completely lost their echinocytic spikes, especially those cells that adopted the most pronounced sickled morphology (Fig 2, IIIB, and C). This process appeared to be completely reversible since these cells immediately readopted their earlier crenated shape upon reoxygenation.

Osmotic deformability profiles of normal cells. The relationship between the deformability index (DI) and the osmolality of the suspending medium for normal red cells is shown (Fig 4A, curve 1). The features of such a profile for a normal red cell are that a maximum value of DI is reached

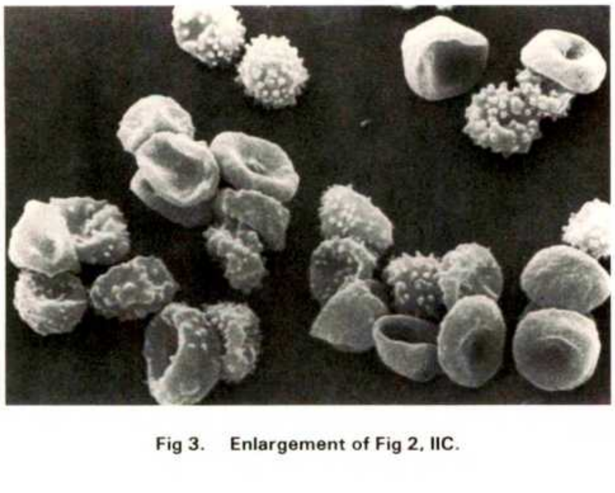

Fig 3. Enlargement of Fig 2, IIC.

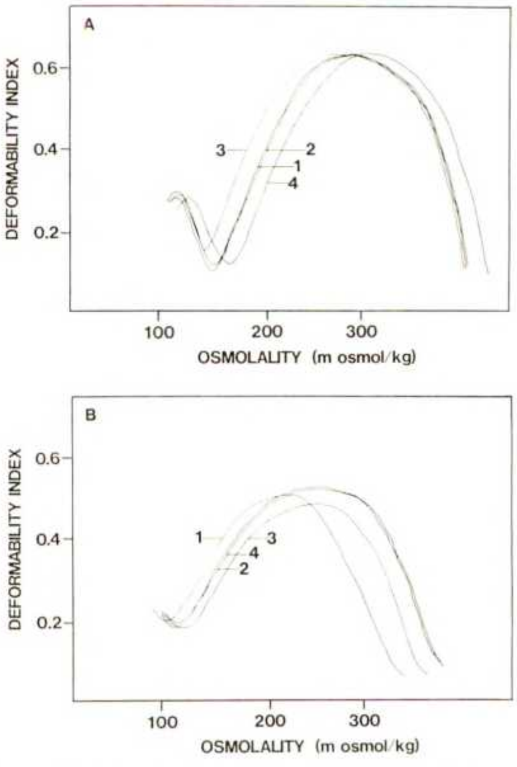

Fig 4. Osmotic deformability profiles of normal RBCs after replacement of their native PC by DPPC (curve 4), POPC (curve 2) and PAPC (curve 3), for 25%, 40%, and 35%, respectively. Curve 1 gives the osmotic deformability profile of control cells. (B) Osmotic deformability profile of sickle cells after replacement of their native PC by DPPC (curve 4), PAPC (curve 3) and POPC (curve 2) for 20%, 30%, and 35%, respectively. Curve 1 gives the osmotic deformability profile of control cells of the same patient. Measurements were performed under oxygenated conditions.

around 290 mosm/kg, while an increase or decrease in tonicity lowers the deformability and therefore the DI of the cell. The decrease in tonicity below 290 mosm/kg lowers the DI until a minimum value around 135 mosm/kg is reached. The osmolality at which this minimum is reached is directly related to the osmolality at which 50% of the cells have lysed in a classical osmotic fragility assay.[15]

The effect of replacement of native PC on the osmotic deformability profile of normal cells is shown in Fig 4A. Replacement of native PC by POPC did not significantly change the osmotic deformability profile of normal red cells (Fig 4A, curve 2), whereas a shift in the deformability profile was observed after replacement of native erythrocyte PC by either PAPC or DPPC (curves 3 and 4, respectively). The profile obtained after replacement with PAPC showed a decrease in the osmolality at which DI reached a minimum in the hypotonic region, while the hypertonic arm of the profile remained unchanged. The maximum attainable value of DI remained constant, but this value was noted over an osmolality range that was broader than that for control cells. Replacement of native PC by DPPC resulted in a profile that showed an increase in the osmolality at which the DI reached its minimum in the hypotonic region. In addition, the hypertonic arm of the curve was also displaced to higher osmolality values. In spite of this shift, the maximum value of DI remained constant, although it was noted over a smaller osmolality range than that seen for control cells.

The alterations in the deformability profile indicate that replacement of native PC with PAPC resulted in a decrease in osmotic fragility of the cells with no change in the state of hydration, whereas replacement with DPPC led to an increased osmotic fragility as a result of cellular hydration. Replacement of native PC with POPC did not significantly change either osmotic fragility or the state of cellular hydration.

Osmotic deformability profiles of sickle cells. Osmotic deformability profiles for native and PC-modified sickle erythrocytes are shown in Fig 4B. The profile of unmodified SS cells (Fig 4B, curve 1) is clearly different from that of normal erythrocytes with much of this difference being accounted for by the heterogeneity in water content of sickle cells.[1] While normal cells were unaffected by subsititution of POPC for native PC (Fig 4A, curve 2), similar treatment of SS cells showed a right shift of the hypertonic arm, indicating increased cell hydration (Fig 4B, curve 2). A similar shift in the osmotic deformability profile was observed with each of the PC species tested (Fig 4B, curves 2, 3, 4) suggesting that PC replacement resulted in increased hydration of sickle cells. While the shifts in osmotic deformability profile found in normal cells seemed to be directly related to the extent of replacement of native PC by PAPC or DPPC this was clearly not the case for sickle cells. Very low levels of replacement of native PC by DPPC (5%) gave a virtually identical shift in the deformability profile as that seen in cells in which 20% of native PC had been replaced by DPPC. Furthermore, while incubation of red cells with PCTP (with or without POPC donor vesicles) had no effect on the osmotic deformability profile of normal RBC (Fig 5A), SS cells showed a shift in their osmotic deformability profile consistent with cellular hydration when incubated with PCTP, with or without POPC donor vesicles (Fig 5B). These results indicate that the presence of PCTP in itself can induce cellular hydration within the heterogeneous sickle cell population.

The concentration dependence and time course of cell hydration following incubation with PCTP is shown in Fig 6. This figure demonstrates the results obtained with red cells from three different patients. Sickle cells become more hydrated with time when incubated in the presence of PCTP (Fig 6A). Maximal hydration induced by 1.1 μmol/L PCTP appeared to occur after 5 to 8 hours incubation (Fig 6B). For a fixed period of time (three hours) the increase in cell hydration, as measured by the shift of the osmotic deformability profile, was found to be dependent on the concentration of PCTP (Fig 6C). Due to the large variations in the initial state of red cell hydration among patients, it is difficult to quantitatively compare the extent of cell hydration induced by PCTP from one patient to another. However, red cell hydration was increased in blood samples from all patients studied.

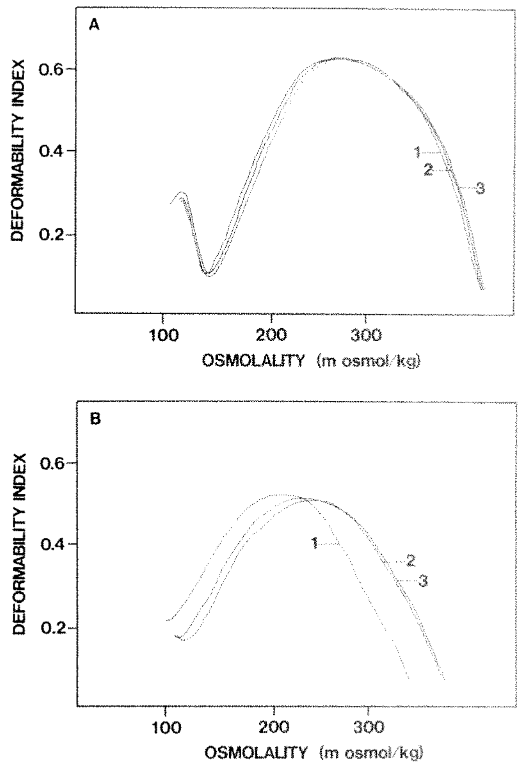

Fig 5. (A) Osmotic deformability profiles of normal RBCs after incubation at 37°C for three hours with 1 μmol/L PCTP in the presence (curve 3) or absence (curve 2) of vesicles containing POPC. Curve 1 gives the osmotic deformability profile after incubation with vesicles in absence of PCTP. (B) Osmotic deformability profiles of sickle cells (patient A) after incubation at 37°C for three hours with 1 μmol PCTP in presence (curve 3) or absence (curve 2) of vesicles containing POPC. Curve 1 gives the osmotic deformability profile after incubation with vesicles in absence of PCTP.

Further evidence for the increased hydration of SS cells was provided by analysis of the buoyant density of sickle cells following incubation with PCTP (Fig 7). Incubation of sickle cells with PCTP led to a decrease in cell density in unseparated sickle cell samples (Fig 7A) as well as in dense sickle cells (Fig 7B).

In order to document whether this hydration of sickle cells induced by PCTP was accompanied by an exchange of PC species between subpopulations of cells, we performed the experiment depicted in Fig 8. Cells with density up to 1.0880 mg/mL were labeled with ^{14}C-PC and ^{51}Cr, as described in the method section. Following incubation in the presence or absence of PCTP, the cells were again separated by buoyant density, and the relative distribution of specific radioactivity over the subfractions was determined (Fig 8). The distribution of ^{51}Cr over the gradient, indicating the buoyant density distribution of the originally labeled cells after the incubation, was virtually identical in the presence (Fig 8A) or absence (Fig 8B) of PCTP. Approximately 60% of the ^{51}Cr label was found in the original position within the gradient (fractions 1 and 2) while the remainder was distributed in higher density fractions (fractions 3 to 6). The distribution of ^{14}C-labeled PC in the density gradient following the incubation in the presence of PCTP showed an even distribution over all cell fractions (Fig 8A), while in the absence of PCTP the distribution of ^{14}C-labeled PC followed the ^{51}Cr distribution (Fig 8B). These results document that exchange of PC

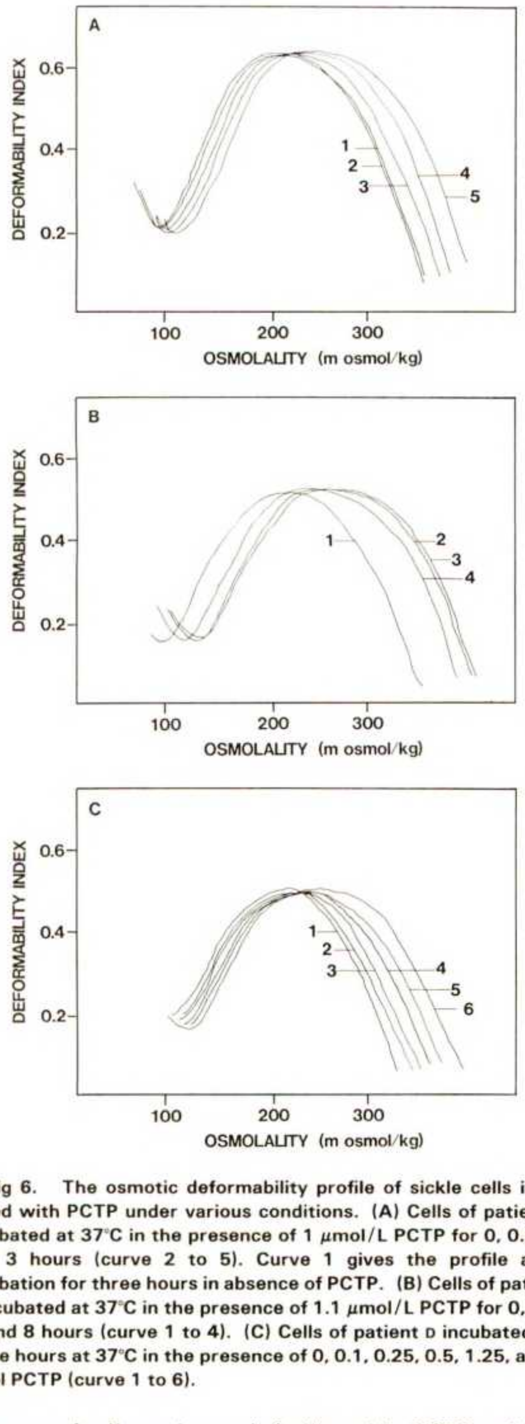

Fig 6. The osmotic deformability profile of sickle cells incubated with PCTP under various conditions. (A) Cells of patient B incubated at 37°C in the presence of 1 μmol/L PCTP for 0, 0.5, 2, and 3 hours (curve 2 to 5). Curve 1 gives the profile after incubation for three hours in absence of PCTP. (B) Cells of patient C incubated at 37°C in the presence of 1.1 μmol/L PCTP for 0, 2.5, 5, and 8 hours (curve 1 to 4). (C) Cells of patient D incubated for three hours at 37°C in the presence of 0, 0.1, 0.25, 0.5, 1.25, and 2 μmol PCTP (curve 1 to 6).

between red cell membranes is facilitated by PCTP and that the observed cell hydration in sickle cells is directly related to this exchange of PC molecules between subpopulations of sickle cells.

DISCUSSION

The PC specific transfer protein from bovine liver is a powerful tool to alter red cell membranes in a well-defined manner. Under proper conditions this protein facilitates exchange of PC molecules between two membranes without altering other aspects of the membrane composition. The present study demonstrates that this exchange process is not limited to PC movement between donor PC vesicles and cells, but that exchange of PC between cell membranes also takes place. In sickle cells as well as normal cells, the rate and extent of PC replacement facilitated by PCTP depends strongly on the PC species involved. These results are in agreement with previously reported results on model systems[17] and erythrocytes.[2,15,18]

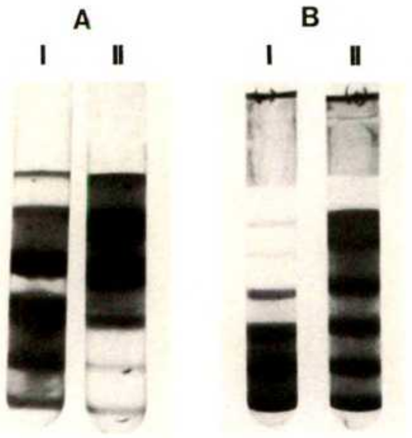

Fig 7. Density dependent separation of sickle RBCs on a discontinuous stractan gradient after incubation for eight hours at 37°C in absence (I) or presence (II) of 1 μmol/L PCTP. (A) Non-separated population of sickle RBCs; (B) dense sickle cells, harvested from a stractan gradient with a density greater than 1.1149 g/mL.

In contrast to normal cells, deoxygenated sickle cells showed a clear difference in the time-dependent replacement of native PC by POPC when compared to oxygenated sickle cells. This observed increase in size of the exchangeable pool of PC in the membrane of sickle cells under nitrogen is in agreement with our earlier observations,[9] and is also an indication that the transbilayer movement of PC in these membranes is accelerated under these conditions.

Since the apolar part of the PC molecules in the membrane can be directly responsible for changes in red cell morphology,[5] retailoring of the molecular species composition of PC might be expected to lead to dramatic changes in cell morphology. The morphologic changes observed in oxygenated sicklable red cells, after replacement of the native PC by DPPC or PAPC, were identical to those observed in normal cells. Sickle cells in which PC was modified changed to a sickle morphology under deoxygenating conditions and at the same time lost their echinocytic appearance. When reoxygenated, the echinocytic shape instantaneously reappeared. This observation is similar to the morphologic changes as a consequence of sickling and desickling of phospholipase A_2-treated RSC's.[8] This loss of echinocytic projections during sickling may indicate a local reorganization of membrane lipids during the sickling process or mechanical stretching of the membrane by the polymerized hemoglobin.

The most striking effect we found in this study was that the ektacytometric deformability profile and state of hydration of the red cell can be affected by retailoring its PC

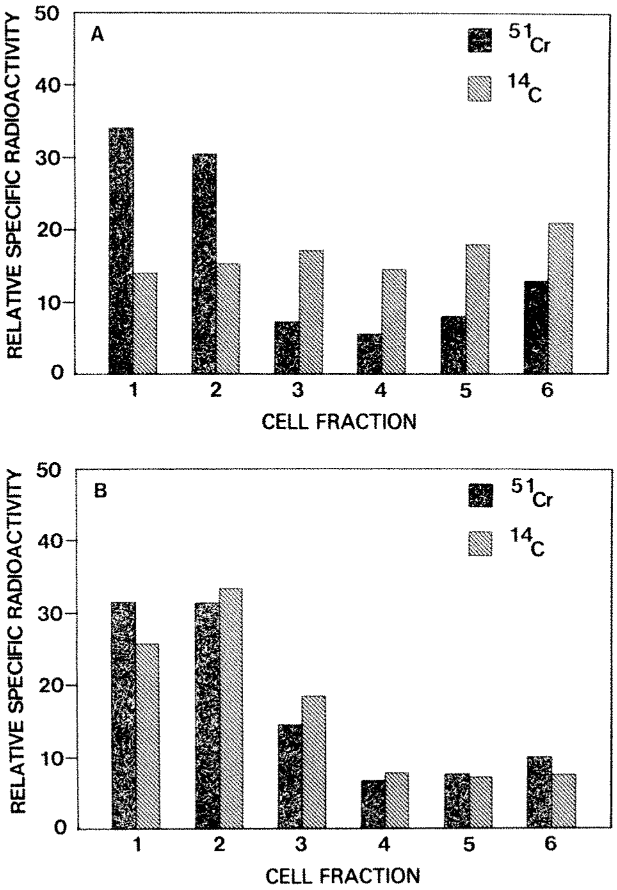

Fig 8. **Exchange of ^{14}C-1-palmitoyl,2-oleoyl PC between subpopulations of sickle cells, in the presence (A) or absence (B) of PCTP. Cells were incubated as indicated in the Materials and Methods section. Cell fractions are denoted as harvested from the stractan gradient from top (1) to bottom (6). Density of stractan gradient layers: (1) <1.0785 g/mL, (2) 1.0785 to 1.0880 g/mL, (3) 1.0880 to 1.0964 g/mL, (4) 1.0964 to 1.1056 g/mL, (5) 1.1056 to 1.1149 g/mL, (6) >1.1149 g/mL. The specific radioactivity of ^{14}C or ^{51}Cr per cell is expressed relative to the total amount found in fractions 1 to 6.**

molecular species. Incubation of normal cells with PCTP in the absence of vesicles or with vesicles in the absence of PCTP had no significant effect on the ektacytometric profile. Replacement of up to 40% of native PC in normal cells by POPC also did not alter the osmotic deformability–profile. However, replacement of native PC by DPPC led to a shift in the osmotic deformability profile indicating an increase in osmotic fragility as a result of cellular hydration. Replacement of native PC by PAPC also shifted the osmotic deformability profile indicating a decrease in osmotic fragility without a change in cellular hydration. Since there was no change in net lipid content and therefore surface area of these red cells, the observed decrease in osmotic fragility is most likely due to a change in membrane permeability. This is consistent with the increase in potassium efflux previously demonstrated under these conditions.[2]

These results indicate that the properties of the normal human red cell in a field of laminar shear stress at varying osmolality can be influenced by a change in the fatty acyl composition of membrane phosphatidylcholine. Neither PCTP nor vesicles by themselves have an effect on these cells as measured by ektacytometry.

In contrast to normal cells, sickle cells demonstrated a shift in the ektacytometric profile, indicating hydration after incubation with PCTP and vesicles with each type of PC species tested. Moreover, incubation of sickle cells with PCTP in the absence of vesicles also induced a change in buoyant density accompanied by an exchange of PC species between individual cells in the sickle cell population. This effect was concentration and time dependent and was found with all patient samples despite individual variations in initial osmotic deformability profile. Our hypothesis for this phenomenon is that PCTP, by facilitating PC molecular–species exchange between membranes or domains within a membrane, induces changes in packing of the molecules in the lipid core, which in turn affects the permeability characteristics of the membrane. Moreover, the replacement of native PC by vesicle PC is not an isolated process since PC exchange between cells takes place as well. The shift in the ektacytometric pattern as shown in Fig 4B is a result of both of these processes. Our results suggest that dehydrated sickle cells lose or gain certain PC species during the exchange process. As a consequence, cell permeability is altered, facilitating cell hydration. Another possibility, one that is less likely but cannot be ruled out at present, is that the PCTP has a unique yet undefined physical interaction with the most dense cells in the SS population and induces a direct change in permeability in these cells. This effect could take place independent of the exchange of PC between subpopulations of sickle cells.

From this study, it can be concluded that changes in the species composition of phosphatidylcholine in normal as well as in sickle cells can lead to an altered cell morphology, membrane permeability, and can affect membrane properties that govern cellular deformability and stability.

A better understanding of the role membrane lipids play in the state of hydration of the sickle cell could be of physiologic significance, since the rate of S hemoglobin polymer formation is extremely dependent on the state of hydration of the cell.

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Bone Marrow Matrix Modulation of HL-60 Phenotype

By Sharon D. Luikart, James L. Sackrison, and Charles A. Maniglia

The initiation and maintenance of cellular differentiation for a variety of cell types has been shown to be influenced by the microenvironment. To investigate the influence of bone marrow stroma on leukemic cell differentiation, HL-60 human promyelocytic leukemia cells were grown in the presence of Triton-treated extracellular matrix derived from normal human bone marrow stromal cells. This bone marrow matrix microenvironment had a dramatic impact on the phenotypic expression of this malignant line. HL-60 cellular proliferation, morphology, nonspecific esterase activity, formation of Fc rosettes, and sensitivity to induction by 12-0-tetradecanoyl-phorbol-13-acetate (TPA) were all influenced by the presence of matrix molecules. In contrast, stromal cell-conditioned media did not alter HL-60 phenotype. Thus, HL-60 cells appear to retain responsiveness to a human bone marrow stromal cell-derived matrix despite their ability to grow autonomously. Studies of the interaction of leukemic cells and marrow stroma in vitro may provide important information concerning the regulation of leukemic cell behavior.

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THE CELLULAR MICROENVIRONMENT plays a major role in the regulation of the differentiation process.[1-4] The interaction of cells with their own extracellular matrix molecules and with those produced by neighboring cell types is an essential factor in the influence of the microenvironment.[5] The basic structural composition of the matrix consists of at least four major classes of macromolecules, including collagen, proteoglycan, elastin, and glycoproteins.[5] These molecules influence the initiation and maintenance of differentiation in a variety of cell types, including myoblasts,[1,6] corneal endothelial cells,[2] kidney epithelial cells,[3] hepatocytes,[4] and neural crest cells.[7] It has been reported that, unlike growth on artificial substrata, maintenance of tissues on more natural matrices can modify cell shape and orientation allowing cells to respond to naturally occurring hormones and growth factors.[8]

One area where the role of the microenvironment has begun to emerge is in hematopoietic cell maturation.[9,10] The homing of transfused marrow cells to recipient marrow and spleen tissue indicates that a supportive matrix is essential to hematopoiesis.[11] Studies suggest that after neoplastic transformation, most cells retain some dependence on growth and differentiation factors.[12] Thus, leukemic cells injected into normal mice can colonize the spleen, but when fresh leukemic cells are injected into Sl/Sl^d mice that have defective stroma, growth is not supported.[13]

Nonetheless, most in vitro studies of human leukemia use cell lines grown in suspension culture. Consequently, few studies have investigated the influence of stroma on leukemic cell development. The studies described in this report examine the interaction of HL-60 human acute promyelocytic leukemia cells with matrix produced by normal human bone marrow stromal cells.

MATERIALS AND METHODS

Cell culture. Heparinized bone marrow samples were obtained from normal volunteer donors on the University of Minnesota Hospital bone marrow transplant service. These patients had a normal hematopoietic status and had not been treated with irradiation or chemotherapy. Bone marrow was diluted with an equal volume of phosphate-buffered saline (PBS), and the red cells were allowed to sediment. Buffy coat cells were cultured in plastic tissue culture flasks containing RPMI 1640 supplemented with 15% heat-inactivated fetal calf serum (FCS) and 50 μg/mL gentamicin (GIBCO; Gaithersburg, MD). An adherent layer of cells was established after several days at 37°C in an atmosphere of air and 5% CO_2, and nonadherent cells were removed. Adherent cells were then grown to confluency, trypsinized, and split 1:4. After several passes, cells were frozen and cultures reinitiated from frozen stocks. These adherent cells had a uniform fibroblast-like appearance and were negative for nonspecific esterase activity.

Human foreskin fibroblasts were isolated by modification of the method of Halaban and Alfano.[14] Foreskins from newborns were cleaned of fat and incubated in 0.25% trypsin at 4°C overnight. The epidermis was separated from the dermis, both tissues were shaken vigorously, and detached cells plated in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% FCS and 1% L-glutamine (GIBCO).

For experiments, adherent bone marrow cells were grown to confluency in 25-cm^2 flasks and maintained in stationary cultures for six additional days. The cultures were then washed with PBS and exposed to 0.5% Triton X-100 (vol/vol) and subsequently washed with PBS to remove remaining nuclei and cytoskeleton elements.[15]

To ascertain the effect of extracellular matrix on cellular phenotype, HL-60 cells were seeded at a density of 1×10^6 cells in 10 mL RPMI/FCS onto matrix, fibronectin (Sigma Chemical Co, St Louis), or uncoated plastic. Fibronectin (10, 100, or 1,000 μg per 25-cm^2 flask) was used as a defined control substrate. For all concentrations, results were similar and given for the 100-μg coating unless otherwise stated. At various times after seeding, cells in suspension were collected, and attached cells were detached by exposure to 2 mmol/L ethylenediaminetetraacetic acid for three minutes at 37°C followed by gentle pipetting. Cell numbers were determined using a model ZF Coulter particle counter (Hialeah, FL). Although <5% of cells were adherent to plastic, ~20% of cells were adherent to fibronectin or bone marrow matrix. In contrast to

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suspension cultures in uncoated flasks, the nonadherent HL-60 cells on stroma appeared to be in close association with the bone marrow matrix, but spontaneously detached with removal of the media.

Assessment of differentiation. After cells were on substrata for 72 hours, suspension and attached cells were combined, and morphological maturation was assessed using Wright-Giemsa staining, nonspecific acid esterase staining[16] and NBT reduction.[17] Induction of Fc rosettes was measured by modification[18] of the method of Bianco et al.[19] Maturation was also assessed in the presence of 1 nmol/L 12-0-tetradecanoylphorbol-13-acetate (TPA; Chemicals for Cancer Research, Inc, Eden Prairie, MN) and various concentrations of retinoic acid (Sigma).

RESULTS

Since uncontrolled proliferation is a characteristic of leukemic pathophysiology, the effects of bone marrow stroma on the rate of proliferation of HL-60 cells in culture were measured (Fig 1). Bone marrow-derived matrix was capable of modifying HL-60 cell proliferation. Thus, after six days in culture, cell numbers decreased by 54% in matrix-coated flasks compared with uncoated flasks. In contrast, fibronectin, the control substrate, had no significant effect on the rate of proliferation.

Concomitant with the dramatic inhibition of neoplastic cell proliferation, bone marrow stroma also induced morphological changes in HL-60 cells. As can be seen in Fig 2, Wright-Giemsa staining of cells grown in stromal cultures exhibits a reduced nuclear:cytoplasmic ratio, irregular cytoplasmic borders, and cytoplasmic vacuoles. Cells grown on fibronectin were similar in morphology to those on plastic, although vacuolization was only rarely observed. Nonspecific acid esterase slides, which were coded and read by independent observers, showed 86% of cells on stroma were positive, while cells on plastic or fibronectin were essentially negative (Table 1). Another parameter of leukocyte maturation is the induction of Fc rosettes.[18] As can be seen in Table 1, cultivation of cells on matrix enhanced induction of Fc rosettes, whereas control substrata did not. However, with 1,000 μg fibronectin, which exceeds the weight of our stroma (10 $\mu g/cm^2$), we did observe that 50% of cells formed rosettes. None of the HL-60 cells on the various substrata demonstrated significant nitroblue tetrazolium (NBT) positivity.

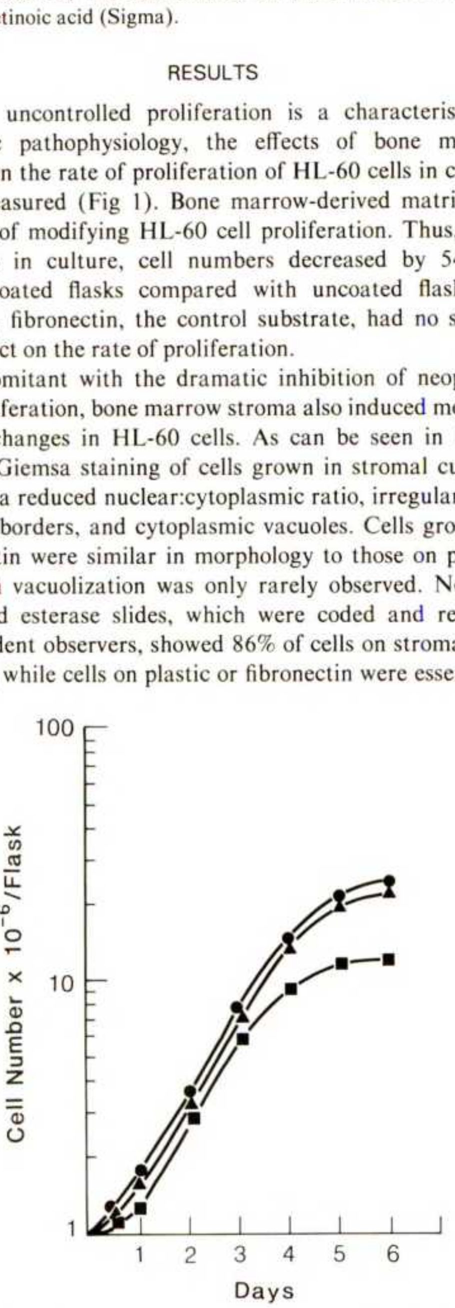

Fig 1. Effect of bone marrow stroma on the replication of HL-60 cells. HL-60 cells were plated at a density of 1 × 10^5 cells/mL on plastic substrata (●), bone marrow stroma (■), or human fibronectin (▲). Each point is the mean of duplicate flasks with less than 10% deviation between flasks.

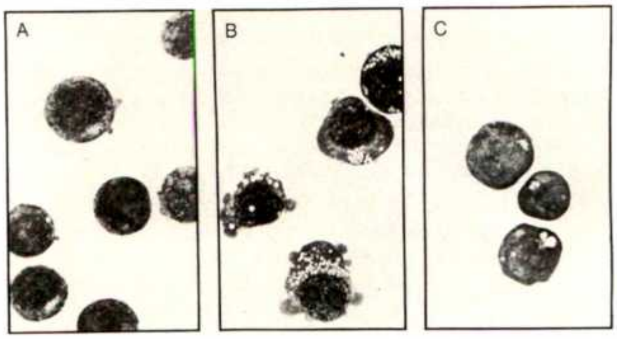

Fig 2. The morphological appearance of HL-60 cells grown on uncoated (A), bone marrow stroma-coated (B), and fibronectin-coated (C) plastic flasks 72 hours after seeding. Slides were prepared by using a Shandon-Elliott cytospin centrifuge (Wright-Giemsa staining: original magnification ×1200; current magnification ×600).

In order to assess the specificity of the bone marrow stroma, HL-60 cells were also grown on matrix derived from human foreskin fibroblasts. HL-60 cells did not adhere to this matrix and, as shown in Table 1, did not display esterase activity. Although there was some vacuolization of the cytoplasm, the cytoplasmic size and borders appeared less mature than cells on bone marrow stroma (data not shown). However, this matrix did enhance rosette formation, as shown in Table 1.

There have been reports of soluble factors that are capable of influencing HL-60 cell behavior.[20] However, as can be seen in Table 1, media conditioned by bone marrow stroma cells did not alter the morphology, esterase production, or rosette formation of HL-60 cells in plastic culture flasks. Thus, free diffusion of the component of the matrix that is regulating HL-60 phenotype did not take place under our culture conditions. However, nonadherent cells in cocultures of HL-60 and stromal cells displayed the morphology, esterase production, and rosette formation seen with HL-60

Table 1. Percentage of HL-60 Cells Expressing Nonspecific Esterase Activity or Fc Rosette Induction

Substrata	Esterase*	Fc Rosettes*
Plastic	7	8
Stroma	86	40
Fibronectin (4 $\mu g/cm^2$)	9	7
Foreskin fibroblast matrix	4	34
Stromal cell-conditioned media	8	13
HL-60/stromal cell coculture media	25	29

*Cells were collected 72 hours after seeding and were assayed. Each value is the average of a minimum of 200 cells scored by two independent observers with <5% variation.

cultures on matrix alone (data not shown). Furthermore, the cell-free media from cocultures was able to alter the phenotype of other HL-60 cells (Table 1). This suggests that the matrix-exposed leukemic cells produced and/or released active factors into the media. Since conditioned media from dimethyl sulfoxide-treated or 1,25-dihydroxyvitamin D_3-treated HL-60 cells has been recently reported to induce monocytic differentiation in fresh HL-60 cells, an autonomously-produced HL-60 differentiation factor has been proposed.[21]

With agents such as dimethyl sulfoxide, HL-60 differentiation is an irreversible process with cessation of proliferation once commitment has occurred.[22] Thus, we wanted to determine whether matrix-induced phenotypic changes were reversible on the removal of the matrix. HL-60 cells were grown for three to six days on matrix or plastic substrate. Cells were then removed and resuspended in fresh media in 25-cm^2 plastic tissue culture flasks. After 24 hours of incubation, the cell number and adhesion were not significantly different in cultures that were initially grown on matrix or uncoated plastic (data not shown). The morphological changes that occurred during exposure to stroma gradually resolved after several passes on uncoated plastic flasks. Thus, these phenotypic changes are dependent on the continued presence of matrix molecules.

A variety of agents can induce HL-60 cells to exhibit a mature phenotype.[23,24] To determine the effect of matrix on HL-60 cell sensitivity to inducers, cells were seeded on plastic or matrix in the presence of 1 nmol/L TPA, an inducer of monocytic differentiation. After 72 or 96 hours, differentiation was assessed. As seen in Table 2, the adhesive response revealed synergistic activity of TPA and matrix. The TPA and matrix cultures also demonstrated greater morphologic maturation with more dramatic cytoplasmic changes and more intense esterase staining than cells on matrix alone. In contrast, this dose of TPA had no effect on cells in uncoated plastic culture flasks. However, the effects of low dose (50, 100, or 200 nmol/L) retinoic acid, an inducer of myeloid maturation, were not enhanced by the presence of matrix.

DISCUSSION

A number of observations support the importance of the microenvironment in the regulation of hematopoietic cell maturation.[9,10,25] Transplanted marrow fibroblasts are able to generate their own specific hematopoietic environment, with those from red marrow forming hematopoietic ossicles and those from yellow marrow forming fatty ossicles.[26] The molecules of the microenvironment may possibly exert their effects on cell phenotype via interaction with the cytoskeleton.[27] For example, it has been suggested that protein synthesis may be affected by changes in arrangement and organization of the polyribosomes that are associated with cytoplasmic microtrabeculae.[28]

Table 2. Percentage of HL-60 Cells Adherent to Substrata

Substrata	Drug	Adhesions*
Plastic	None	5
	1 nmol/L TPA	5
Stroma	None	25
	1 nmol/L TPA	50

*Cells were counted 96 hours after seeding. Each value is the mean of duplicate flasks with <5% deviation between flasks, representing one of four experiments with similar results.

The influence of the microenvironment on neoplastic cells has been reported for a variety of malignant cell types.[29-31] In hematological malignancy, long-term cultures infected with Friend murine leukemia virus produce immature cells that produce leukemia in recipient mice. However, these cells retain their dependency on the marrow-adherent layer for in vitro growth.[32] The HL-60 cell line grows in suspension culture and does not require growth factors for long-term maintenance, but our results demonstrate that expression of a mature phenotype by HL-60 cells can be influenced by the presence of the marrow stroma. Similarly, Steinberg et al[33] reported an increase in NBT-positive colonies when HL-60 cells were grown in coculture with Dexter cultures of human bone marrow compared to cultures without stromal cells. These investigators suggested that cell-cell contact mediated the effect. In addition, Schölzel and Löwenberg[34] reported expression of mature leukocyte surface antigens by freshly-isolated myeloblasts grown in coculture with adherent cells from a normal marrow. In that study morphologic maturation was incomplete, and leukemic cell replication was stimulated compared with cultures without stroma. However, our results were not dependent on intact stromal cells.

The marked differences in HL-60 cells grown on marrow stroma imply these myeloid leukemia cells, despite their autonomous growth in vitro, retain responsiveness to the environment. Other examples of myeloid leukemic cells that no longer require growth factors but are sensitive to physiological inducers of differentiation have been reported.[35] Other malignant cells have loss of additional controls leading to blocks in differentiation, but even these cells may differentiate in the presence of nonphysiological agents that use other maturation pathways.[36] This uncoupling of growth and maturation regulation has been suggested as a cause of leukemia.[36]

The change in sensitivity of HL-60 cells to the monocytic differentiation inducer, TPA, in the presence of matrix may be quite important in considering future in vitro screens for therapeutic efficacy. Similarly, Gospodarowicz et al[37] found that corneal epithelial cells maintained on collagen were sensitive to epidermal growth factor, but those grown on plastic were not. We plan to investigate further the effect of bone marrow matrix on the sensitivity of myeloid leukemic cells to a variety of potential differentiation agents.

The precise role of the various components of the complex hematopoietic microenvironment in regulation remains to be determined. In long-term cultures in which adherent stromal cells support hematopoiesis, growth-regulating proteins are not found in large quantities in the media.[38] In the experiments reported here, stromal cell-conditioned media did not alter HL-60 phenotype. Thus, the importance of cell interactions and local control over hematopoietic cell development as opposed to diffusible molecules has been recognized.[38] Whether or not the phenotypic changes seen here are due to a local concentration of regulatory proteins or other components of extracellular matrix (collagen, laminin, or proteoglycan) is under current investigation in our laboratory. Gordon et al[39] recently reported that GM-CSF binds to glycosaminoglycan components of the bone marrow matrix leading to selective compartmentalization. However, studies of the effects of recombinant human GM-CSF and G-CSF on HL-60 cells demonstrated no morphological maturation, although membrane antigen expression was altered.[40] Our results also suggest that various characteristics may be influenced by different components, since rosette formation is much less specific than the other markers we have used. Investigations of the phenotype of HL-60 cells grown on defined matrices and on partially degraded marrow stroma are currently underway.

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Immunohistochemical Characterization of a 183 KD Myeloid-Specific–DNA-Binding Protein in B5 Fixed, Paraffin-Embedded Tissues, and Bone Marrow Aspirates by Monoclonal Antibody BM-1

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A monoclonal antibody, designated BM-1, which is reactive in B5 formalin-fixed, paraffin-embedded tissues, has been generated against a cytoplasmic and nuclear antigen expressed in human myeloid precursor cells and derived leukemias. Using the avidin-biotin-complex immunoperoxidase procedure, BM-1 was found to stain selectively myeloid precursor cells in normal bone marrow and mature granulocytes in the blood. In a screen of 26 normal adult and fetal human organs fixed in B5 formalin, BM-1 was negative in all nonhematopoietic tissues with the exception of tissue granulocytes and scattered cells in the peripheral cortex of the thymus. Likewise a screen of 30 solid tumor cell lines including a spectrum of carcinomas, sarcomas, and neural-derived tumors was negative. BM-1 was also negative with 21 T and B cell lymphomas and 11 Hodgkin's disease tumors. A preliminary study of tumors of the hematopoietic system revealed that BM-1 was reactive with M2 and M3 acute myelogenous leukemias (AML), chronic myelogenous leukemias (CML) and myelomonocytic leukemias, and granulocytic sarcomas. M1, M4, M5, and M6 AML clot preparations were negative in this study, indicating that BM-1 may have a role in the histopathologic diagnosis of myelogenous leukemia. Myeloid leukemic cell lines HL-60, ML-2, KG1, and TPH-1-0 showed BM-1 nuclear and/or cytoplasmic reactivity in a subpopulation of cells, but erythroid and lymphoid leukemias and all lymphoma cell lines were negative. Immunoperoxidase studies of a panel of fetal tissues showed BM-1 positive cells in the peripheral cortex of the thymus and portal myelopoietic regions of the liver at 18 weeks gestation. Finally, DNA-cellulose and solid phase radioimmunoassay (RIA) techniques developed in our laboratory demonstrate that the BM-1 antigenic domain is reactive only after binding to eukaryotic but not prokaryotic single- or double-stranded DNA. Immunoblot techniques using a DNA-cellulose purified protein sample revealed that BM-1 recognizes a 183 kD protein. These studies indicate that BM-1 is recognizing a myeloid-specific antigen that, because of its DNA binding characteristics, may have an important role in the differentiation of myeloid cells at the molecular level.

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A LARGE NUMBER of monoclonal antibodies to myeloid-specific–cell-surface antigens have been described.[1-23] Some of these reagents have proven useful as markers of myeloid differentiation and have greatly improved our ability to characterize and diagnose acute myelogenous leukemia (AML) subtypes.[24-33] In a previous report[34] we described the generation of three monoclonal antibodies (MoAbs) to myeloid-specific nuclear antigens expressed in differentiating human myeloid leukemia cells and demonstrated that MoAbs to internal cellular antigens may be both cell- and maturation-specific in nature. In the present study a new myeloid-specific MoAb, designated BM-1, is described, which is directed against a DNA-binding protein expressed in the cytoplasm and nucleus of normal and malignant myeloid cells. Unlike other reported markers, BM-1 appears to be restricted in its reactivity to M2 and M3 AML as identified by the French-American-British (FAB) classification.[35] In addition, BM-1 retains its immunoreactivity in B5-fixed, paraffin-embedded tissue sections, including those prepared from bone marrow aspirate clot preparations, and therefore appears to have diagnostic utility as a tumor marker for myeloid leukemias and granulocytic sarcomas. Because of its restricted expression in M2 and M3 AML, BM-1 may identify a maturation-specific antigen that can be used to study the differentiation and transformation of human myeloid cells.

MATERIALS AND METHODS

Cell lines and tissues. A complete list of the lymphoid and solid tumor cell lines used in these experiments is shown in a previous publication.[36] Tumor cell lines designated with the SW prefix were obtained from Dr William McCombs, III, at the Scott and White Memorial Hospital, Temple, Texas. All of the cell lines were grown in RPMI 1640 medium containing 10% fetal calf serum (FCS), 100 U/mL penicillin-G, and 100 μg/mL streptomycin sulfate. The cell lines were cultured in a well-humidified 5% CO_2 incubator and were routinely passaged twice per week.

For the immunohistochemical studies, human tissues fixed with B5 formalin were obtained from biopsies performed on patients for diagnostic procedures at the Los Angeles County-University of Southern California Hospital and Northwestern Memorial Hospital. Fetal tissues (gestational ages 18 to 22 weeks) obtained from abortuses were fixed in B5 formalin prior to paraffin-embedding procedures. Bone marrow aspirates from leukemia and lymphoma patients were prepared as clot preparations and fixed in B5 formalin prior to paraffin-embedding procedures. All patients participating in this study were advised of procedures and attendant risks in accordance with institutional guidelines and gave informed consent.

Antigen preparation. Nuclei from human peripheral blood mononuclear cells were prepared as previously described.[37]

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Immunization protocol. One-milliliter aliquots of the nuclear preparations were thawed, sonicated to reduce viscosity, and emulsified in 1.5 mL of complete Freund's adjuvant by using two glass syringes and a 20-gauge microemulsifying needle (Biolab, Inc, Lake Havasu City, AZ). Three 10-week-old Balb/c female mice were injected subcutaneously (SC) at multiple sites by using a 22-gauge needle and glass syringe. Two weeks later the mice were reinoculated as above, except the nuclear extracts were prepared in incomplete adjuvant. Ten days later the mice received a third inoculation of antigen, this time without adjuvant and by intravenous (IV) injection. Four days later the mice were killed by cervical dislocation and the spleens removed by aseptic techniques.

Cell fusion and cloning procedures. Spleen cells were fused with 8-azaguanine-resistant mouse myeloma NS-1 cells at a ratio of 5:1 respectively by using 40% polyethylene glycol 1540 MW (J.T. Baker, Phillipsburg, NJ), as described by Frazekas de St Groth and Scheidegger.[38] Culture supernatants from wells with active cell growth were tested by indirect immunofluorescence microscopy with fixed-cell preparations as described below. Positive cultures were cloned on 0.5% Noble agar (Difco Laboratories, Detroit) containing RPMI 1640 medium, 20% FCS, and antibiotics.[39]

Serologic characterization of MoAb isotypes. Hybridoma supernatants from four-day cultures were concentrated 20 times in B15 Minicon concentrators (Amicon, Lexington, MA) and tested in double-diffusion Ouchterlony plates against rabbit antimouse immunoglobulin–heavy chain-specific antisera (Miles Laboratories, Inc, Elkhart, IN). The precipitin bands were read after two days incubation at 37°C.

Fixed cell indirect immunofluorescence. To examine cells for the presence of intracellular antigens, paraformaldehyde-acetone–fixed cell preparations were used. The preparation of these slides as well as the indirect immunofluorescence methods have been described in detail previously.[40]

Immunoperoxidase staining. Frozen and paraffin sections prepared from B5 formalin-fixed human tissues were stained with MoAb BM-1 using the avidin-biotin complex–immunoperoxidase staining kit (Vector Laboratories, Inc, Burlingame, CA) as described by Hsu et al.[41] For these experiments a one-fourth dilution of BM-1 supernatants was used. As a negative control MoAb 43H3 (IgG_1), which is unreactive in paraffin-embedded sections, was used with each run.

DNA-cellulose and DNA solid-phase radioimmunoassays. The chemical nature of the BM-1 antigen was further studied using two assays developed in our laboratory to identify MoAbs to DNA-binding proteins. For these experiments three myeloid-specific–antinuclear MoAbs (825-18, 882-17, and 888-5) were used for comparison. The binding characteristics of these reagents have been described elsewhere.[34] In the first method single-stranded and double-stranded DNA celluloses were prepared by the procedure of Moss et al[42] using calf thymus DNA (Sigma Chemical Co, St. Louis), as previously described.[43] Cellular lysates from antigen positive and negative cell lines or biopsies were prepared in Ca/PIPES buffer (0.5 mmol/L $CaCl_2$, 1×10^{-4} mol/L 1,4-Piperazinediethanesulfonic acid, pH 6.5, 0.5 mmol/L phenylmethylsulfonyl fluoride) using brief sonification. After clarification by ultracentrifugation at 30,000 rpm for 30 minutes at 4°C, 1-mL aliquots of lysate were incubated overnight at 4°C with 250 μL of either single- or double-stranded DNA cellulose using constant vortexing (The Big Vortexer, Kraft Apparatus, Inc, Mineola, NY). After washing the DNA-cellulose twice with wash buffer (0.15 mol/L NaCl, 0.1 mol/L Tris, pH 8.0), each tube was incubated at 4°C for one hour with 1 mL of MoAb supernatant followed by two washes and a final one-hour incubation at 4°C with 100,000 cpm of ^{125}I-labeled goat antimouse IgG (Amersham, Inc, Arlington Heights, IL). The DNA-cellulose was then washed four times and counted in a gamma counter. Each reaction was tested in triplicate to determine the statistical significance of the data.

In the second assay DNA from various sources were tested for their capacity to bind the BM-1 antigen. Using a solid phase radioimmunoassay (RIA), 2.5 μg of DNA from calf thymus, *Escherichia coli*, and pBR322 plasmids were attached to polystyrene multiwell strips (Dynatech Inc, Alexandria, VA) overnight at 4°C. After two rinses with wash buffer (1 mg/mL bovine serum albumin in phosphate-buffered saline [PBS]), the wells were blocked for one hour with 10 mg/mL bovine serum albumin in PBS at 4°C. The wells were then incubated with 50 μL MoAb supernatant for one hour at 4°C. After six washes the wells were finally incubated with 100,000 cpm of ^{125}I-labeled goat antimouse IgGs for one hour at 4°C. The wells were then washed 6 times and counted in a gamma counter.

Immunoblot procedure. A chronic myelogenous leukemia (CML) biopsy that expressed a high level of the BM-1 antigen, as determined by DNA cellulose RIA, was used in an immunoblot procedure to identify the mol wt of the BM-1 antigen. Other MoAbs with known reactivities were used as controls to verify the specificity of the antigen–antibody reactions. Whole cell lysates were dissolved in nonreducing sodium dodecyl sulfate (SDS) sample buffer and electrophoresed in 5% to 10% SDS-polyacrylamide gradient gels at 20 mA for four hours. Alternatively, myeloid cell lysates produced in Ca/PIPES buffer as described above were prepurified on DNA-cellulose using FPLC chromatography (Pharmacia, Piscataway, NJ). Details of this procedure will be presented in a separate publication. Antigen-containing fractions were identified by DNA-cellulose RIA, as described above. Positive fractions were combined and concentrated by ultrafiltration using 25,000 mW cutoff Centriflo membrane cones (Amicon, Danvers, MA) before being electrophoresed using the same conditions described above. The separated proteins were transferred to nitrocellulose (Millipore grade HA, 0.45 μm) using a semidry electroblot apparatus (Sartorius, West Coast Scientific Co, Inglewood, CA) in transfer buffer (24 mmol/L Tris-HCl, pH 8.3, 192 mmol/L glycine, 20% methanol) at 160 mA for three hours at room temperature. Lanes containing the whole cell lysate and mol wt markers (BioRad, Richmond, CA) were stained for ten minutes with 0.1% amido black and destained in 10% glacial acetic acid and 5% methanol. The remaining lanes were blocked with 0.25% Tween-20 in PBS for one hour at room temperature and washed in 0.05% Tween-20 in PBS (wash buffer). The lanes were then incubated for one hour with BM-1 supernatant (neat) and washed 3 times with wash buffer for 10-minute intervals. Five micrograms of biotinylated goat antimouse IgG (Vector Laboratories, Burlingame, CA) diluted in 1 mg/mL bovine serum albumin in PBS was then added for one hour and then washed as above. After this step the lanes were incubated with avidin-biotin-complex (Vector Laboratories) using 100 μL of each reagent in 0.3 mol/L NaCl for 30 minutes followed by three washes. Finally, the lanes were developed with 6 mg 4-chloro-1-napthol (Sigma Chemical Co, St Louis, predissolved in 2 mL methanol) diluted in 10 mL PBS containing 5 μL of 30% H_2O_2 for 15 minutes followed by repeated washings.

RESULTS

Generation of MoAb BM-1. Hybridoma clone BM-1 was produced by the fusion of mouse myeloma NS-1 cells and Balb/c splenocytes obtained from a mouse hyperimmunized with nuclei from human peripheral blood mononuclear cells. Isotypic analysis revealed that MoAb BM-1 is of the IgG_1 heavy chain subclass. BM-1 was initially identified by indirect immunofluorescence techniques with the use of

Table 1. Reactivity of BM-1 With Human Myeloid Leukemia Cell Lines by Indirect Immunofluorescence

Cell Line	BM-1*
HEL92.1 (erythroid)	—†
K562 (erythroid-CML)	—
HL-60 (promyelocytic)	+ 5% cytoplasmic, 5%-20% nuclear
KG1 (myeloid)	5% nuclear
ML-2 (myeloid)	+
TPH-1-0 (monocytic)	+ 10% nuclear

*Fixed-cell indirect immunofluorescence assay.

†Data expressed as (−) negative; (+) positive.

paraformaldehyde-acetone–fixed cell preparations from peripheral blood.

Table 2. Reactivity of BM-1 With Human Leukemias in B5-Fixed, Paraffin-Embedded Clot Preparations

Leukemia	BM-1
AML	
M1	0/7*
M2	6/8
M3	2/2
M4	0/4
M5	0/3
M6	0/2
ALL	0/12
Chronic Leukemia	
CML	2/2
CMMOL	3/3
CLL	0/4
Multiple myeloma	0/4

*Number of positive cases/total number of cases.

Indirect immunofluorescence studies with established human tumor cell lines. Indirect immunofluorescence studies revealed that BM-1 was negative with all T, B, and undifferentiated lymphomas and leukemias, myeloma cell lines, and Epstein-Barr virus (EBV)-transformed lymphoblastoid cultures. In addition, BM-1 was negative with 30 solid tumor cell lines derived from a wide variety of human tumor types. As shown in Table 1, BM-1 stained a small percentage of HL-60, ML-2, KG1, and TPH-1-0 cells. The erythroleukemia cell lines, however, were negative by these procedures. These results indicate that BM-1 is specific for myeloid leukemia cell lines where the antigen is expressed in a small percentage of cells, perhaps at a discrete period of maturation or growth.

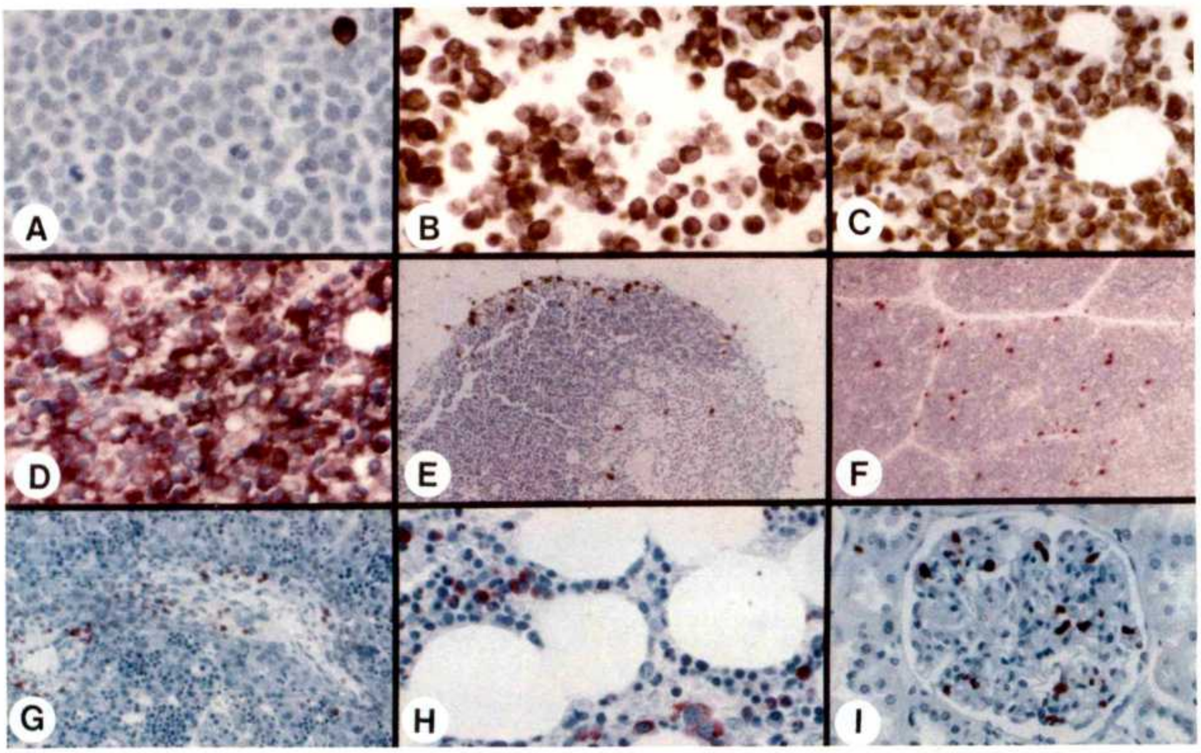

Fig 1. Immunoperoxidase staining reactivity of MoAb BM-1 on B5-fixed, paraffin-embedded tissue and bone marrow aspirate clot sections. (Counterstained with hematoxylin). (A) BM-1 staining of M1 AML bone marrow aspirate clot preparation showing no reactivity with leukemic population. A BM-1 positive cell in upper right hand corner may be a residual, normal, myeloid precursor cell and acts as an internal positive control for this specimen (magnification ×936). (B) BM-1 staining of M2 AML bone marrow aspirate clot section showing strong reactivity of BM-1 with leukemic cells (magnification ×835). (C) BM-1 staining of CML bone marrow clot section showing strong positive staining of the majority of leukemic cells (magnification ×793). (D) BM-1 staining of granulocytic sarcoma biopsy showing strong cytoplasmic staining of malignant cells (magnification ×790). (E) BM-1 staining of adult thymus revealing antigen-positive cells at the periphery of the cortex (magnification ×124). (F) BM-1 staining of fetal thymus again revealing BM-1 positivity at the peripheral cortex and in the interlobular spaces (magnification ×122). (G) BM-1 staining of fetal liver identifying sites of early myelopoiesis at the portal areas (magnification ×266). (H) BM-1 staining of normal adult bone marrow showing selective staining of myeloid precursor cells around fat globules (magnification ×720). (I) BM-1 staining of adult kidney with a granulocytic infiltrate in the glomerulus (magnification ×403).

Table 3. Reactivity of BM-1 With Human Lymphomas and Related Diseases in B5-Fixed, Paraffin-Embedded Tissues

Lymphoma* or Disease	BM-1
WDLL	0/2†
PDL	0/4
Mixed	0/4
DHL	0/6
Undifferentiated (Burkitt's)	0/1
T cell	0/2
Mycosis fungoides	0/2
Hodgkin's disease	0/11
CML in spleen	1/1
Gaucher's disease	0/1
Granulocytic sarcoma	2/2

Abbreviations: WDLL, Well-differentiated lymphocytic lymphoma; PDL, poorly-differentiated lymphocytic lymphoma; mixed, mixed lymphocytic and histiocytic lymphoma; DHL, diffuse histiocytic lymphoma.

*Rappaport classification.

†Number of positive cases/total number of cases.

Immunoperoxidase studies using B5-fixed, paraffin-embedded tissues from human leukemias, lymphomas, and normal biopsies. The immunoperoxidase staining reactivity of BM-1 on B5-fixed, paraffin-embedded clot preparations from acute and chronic leukemias and multiple myelomas is presented in Table 2. In the acute leukemias 8/10 cases with M2 and M3 subtypes were positive, while the other subtypes were negatively stained. All five cases of CML were strongly positive, while those derived from lymphoid cells were uniformly negative. Acute lymphocytic leukemia (ALL) and myeloma were also negative. The staining reactivities of BM-1 with M1 and M2 AML and CML cases are shown in Fig 1A, B, and C respectively. From these studies BM-1 was found to stain the cytoplasm of the leukemia cells, and only minor staining of the nucleus was noted in some cases.

In Table 3 the reactivity of BM-1 on human lymphomas and related diseases is shown. All 32 cases of Hodgkin's and non-Hodgkin's lymphomas were negative for BM-1. In contrast, the CML case in the spleen was strongly positive, as were the two cases of granulocytic sarcoma. The staining reactivity of BM-1 with one of the granulocytic sarcoma cases is shown in Fig 1D.

The staining reactivity of BM-1 with normal lymphoid and hematopoietic tissues in B5-fixed, paraffin-embedded tissues obtained at biopsy showed that, as expected, BM-1 was negative in lymph node, tonsil, and adult thymus except for the presence of scattered and variable numbers of polymorphonuclear leukocytes and for a few cells at the periphery of the cortex of the thymus, shown in Fig 1E. In fetal thymus shown in Fig 1F, there was an increased number of BM-1 positive cells in the peripheral cortex and in the interlobular spaces. The origin of these cells is unknown, but it is possible that they represent myeloid precursors in areas of potential myelopoiesis. Similarly, BM-1 positive cells were found in portal regions of fetal liver at 18 weeks gestation (Fig 1G) identifying sites of early myelopoiesis. Splenic biopsies showed strong reactivity with granulocytes. Of interest, monocytes in peripheral blood specimens were negative. As shown in Fig 1H, clusters of myeloid precursors in the bone marrow showed strong cytoplasmic reactivity, but erythroid and megakaryocyte precursors were negative.

The immunoperoxidase staining reactivity of BM-1 with normal adult and fetal nonhematopoietic tissues fixed in B5 showed that except for scattered tissue granulocytes and areas of myelopoiesis mentioned above, all of these tissues were negative, providing further proof of the myeloid specificity of BM-1. In Fig 1I an example of the tissue granulocyte reactivity of BM-1 is shown in a section of kidney.

Finally, the immunoreactivity of BM-1 was shown to be variable in tissues using formalin and other nonprecipitating fixatives. Decalcification of bone marrow biopsies also showed variable staining by BM-1, indicating that this step is potentially detrimental to BM-1 reactivity. Cryostat sections of human tissues and leukemic samples, however, showed the same staining intensity and pattern of reactivity as B5-fixed tissue specimens for BM-1.

Biochemical characterization of the BM-1 antigen. Routine immunoblotting procedures using myeloid whole-cell lysates failed to identify the mol wt of the antigen recognized by BM-1. However, as shown in Table 4, DNA-binding RIAs using single- and double-stranded calf thymus DNA and a cellular lysate prepared from CML cells showed that BM-1 recognizes a DNA-binding protein. Lysates from nonmyeloid sources produced negative results, as did a panel of 30 antinuclear MoAbs generated in our laboratory (data not shown). For comparison, myeloid-specific antinuclear MoAbs 825-18, 882-17, and 888-5[34] and NS-1 control supernatants were run in parallel with BM-1 and showed no positivity in this assay with the CML lysate. DNA from prokaryotic sources also failed to support BM-1 binding in the presence of the CML lysate. Identical results were obtained using both DNA-cellulose and solid-phase RIAs. These data strongly support the interpretation that BM-1 identifies a DNA-binding protein whose active antigenic site is present when bound to eukaryotic DNA. As shown sche-

Table 4. DNA-Binding RIAs

	DNA Cellulose RIA					Solid Phase RIA						
	Lymphoid Lysate		Myeloid Lysate			Lymphoid Lysate		Myeloid Lysate				
MoAb	+SS	+DS	−DNA	+SS	+DS	+SS	+DS	−DNA	+SS	+DS	+E coli/DNA	+PBR322/DNA
BM-1	−*	−	−	++	++	−	−	−	++	++	−	−
825-18	−	−	−	−	−	−	−	−	−	−	−	−
882-17	−	−	−	−	−	−	−	−	−	−	−	−
888-5	−	−	−	−	−	−	−	−	−	−	−	−
NS-1	−	−	−	−	−	−	−	−	−	−	−	−

Abbreviations: +SS, with single-stranded calf thymus DNA; +DS, with double-stranded calf thymus DNA.

*Data expressed as − : background cpm; + : 5× background cpm; ++ : 10× background cpm.

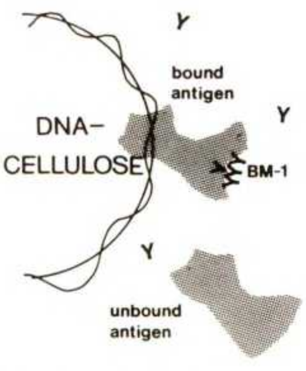

Fig 2. Diagrammatic sketch of DNA-cellulose RIA offering one interpretation of experimental results. In this assay myeloid cell lysates were reacted with single- or double-stranded eukaryotic DNA bound to cellulose beads followed by BM-1 binding. A ^{125}I-goat antimouse IgG secondary antiserum was then used to quantitate the amount of BM-1 bound to the DNA-cellulose beads. Upon binding of the BM-1 antigen to the DNA, the antigenic site becomes accessible, possibly by a conformational change in the molecular structure as shown here. Free p183 in solution is not in the right conformation and is therefore not able to bind to BM-1.

matically in Fig 2, BM-1 apparently recognizes a myeloid-specific protein that upon binding to single or double stranded DNA changes its antigenic structure to an active form. It will be of interest to determine if this DNA-binding protein recognizes specific sequences of DNA that may be related to myeloid differentiation. Attempts to identify the BM-1 antigen on nitrocellulose eventually were successful after DNA-cellulose-purified–BM-1 positive fractions of myeloid lysates were used in the immunoblot experiments. As shown in Fig 3, BM-1 antigen that was eluted from the FPLC DNA-cellulose column was shown to be a 183,000-Dalton protein. Although DNA was not present in these preparations, it is presumed that a small percentage of p183 was in the right configuration for BM-1 binding to occur on the immunoblots.

DISCUSSION

A new MoAb that reacts strongly in B5-fixed, paraffin-embedded tissue sections has been produced to a human myeloid antigen. The salient characteristics of BM-1 are summarized in Table 5. In general BM-1 is highly specific to myeloid precursor cells of the bone marrow, to circulating and tissue infiltrating granulocytes, and in these preliminary studies to M2 and M3 AMLs, CMLs, and granulocytic sarcomas. The antigen identified by BM-1 appears to be localized mainly to the cytoplasm in B5-fixed tissues but is also expressed in the nucleus, as demonstrated by indirect immunofluorescence techniques using paraformaldehyde-acetone–fixed human myeloid leukemia cell lines. To date no significant staining has been observed in nonmyeloid cells, including those from a wide spectrum of normal and malignant human tissues and cell lines.

Finally, DNA-cellulose RIA studies have convincingly shown the BM-1 antigen to be a myeloid-specific–DNA-binding protein. Because of its DNA-binding properties, the BM-1 antigen was able to be purified by DNA-cellulose affinity chromatography using novel FPLC techniques, to be described elsewhere. Although initial attempts to immunoblot myeloid whole-cell lysates with BM-1 were unsuccessful, later experiments using partially purified DNA-cellulose–affinity-chromatographic fractions revealed that BM-1 recognizes a 183-kD protein. Further studies are underway to identify the nature of the BM-1 antigen and to characterize further its specificity for DNA.

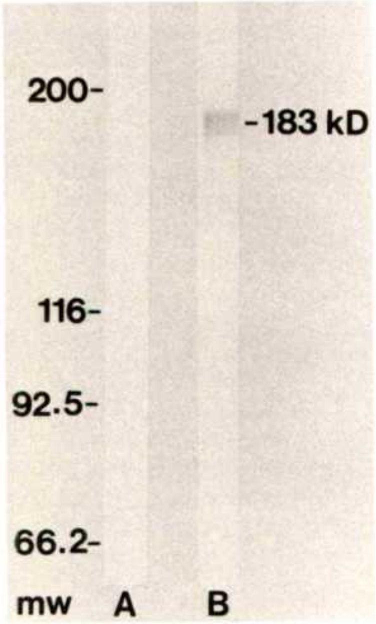

Fig 3. Immunoblot results with MoAb BM-1 against (A) unreduced whole-cell lysate of CML cells and (B) DNA-cellulose (prepurified) unreduced antigen from CML lysate. BM-1 recognizes a 183-kD protein by this method.

From these results, three major findings have emerged. First, we have shown that MoAbs that retain their reactivity in B5-fixed, paraffin-embedded tissue sections can be produced to myeloid subsets. The ability of BM-1 to stain paraffin sections was dependent upon the use of B5 fixation, since nonprecipitating formalin fixatives gave only variable results. Like the LN-1 and LN-2 MoAbs to human lymphoid subsets previously described by our laboratory,[36] the BM-1 antibody retains its reactivity in paraffin, presumably because the B5 fixative protects the antigenic determinants from denaturation or masking during the tissue-embedding process. The immunoperoxidase staining patterns of BM-1 in

Table 5. Characteristics of MoAb BM-1

Immunogen	Peripheral blood mononuclear cell nuclei
Isotype	IgG_1
Antigen	183-kD DNA-binding protein
Antigen site	Cytoplasm and nucleus
Bone marrow reactivity	Myeloid precursors
Lymphoid reactivity	None
Adult and fetal tissue reactivity	Tissue granulocytes; scattered cells in peripheral cortex and interlobular septae of adult and fetal thymus; portal regions of fetal liver (18 weeks).
Tumor specificity	M2, M3, AML; CML and CMMOL; granulocytic sarcoma

human tissues and leukemic samples have been confirmed in cryostat sections, signifying that the staining reactivity of BM-1 in paraffin sections is consistent with that of other methods of tissue preparation. Because of its ability to stain paraffin-embedded tissue sections and its apparent specificity for myeloid leukemias, BM-1 may have diagnostic potential in the identification of granulocytic sarcomas. As discussed by Whitcomb et al,[44] the diagnosis of granulocytic sarcoma may be difficult and may be confused with lymphoid malignancies. A specific marker, such as BM-1, for myeloid-derived tumors that retains its reactivity in routine pathologic specimens would be an important new tool for the diagnostician.

The second major finding is that BM-1 appears to recognize a novel myeloid-specific maturation antigen that is not expressed in other human tissues or malignancies. Because of its cytoplasmic and nuclear localization, its ability to stain paraffin-embedded tissues, and its DNA-binding properties, the 183-kD BM-1 antigen is readily distinguishable from other reported myeloid-associated antigens. Earlier studies by Goldberger et al[45] and Briggs et al[46] have revealed the presence of myeloid-associated–nonhistone nuclear antigens using polyclonal antibodies to dehistonized chromatin preparations. Likewise Whittingham et al[47] identified a granulocytic nuclear antigen using sera from patients with rheumatoid arthritis and autoimmune, chronic, active hepatitis. More recently our own laboratory reported on the characterization of three MoAbs to myeloid-specific nuclear antigens and showed that these antigens were expressed during different periods of cell maturation after induction of myeloid leukemia cell lines with 1,25 dehydroxyvitamin D_3 and dimethylsulfoxide.[34] Finally, Fitz-Gibbon et al[48] have used MoAbs to identify cytoplasmic antigens associated with granules expressed in myeloid cells. All of these studies indicate that myeloid-specific antigens exist not only on the cell surface but also within the cytoplasm and nucleus and that these antigens may be useful in characterizing myeloid maturation. Nuclear antigens, in particular, may be associated with DNA sequences or RNA processing within the nucleus required for the expression of myeloid-specific antigens. In this context the BM-1 antigen, which has been shown to have DNA-binding properties, is worthy of further study, and its mode of action within the nucleus may represent an important model of cellular differentiation.

The third major result of this study is that BM-1 appears to be restricted in its immunoreactivity to M2 and M3 AML. Although other combinations of MoAbs have been shown to have subset specificity,[27] BM-1 alone has this specificity among the acute leukemias. Our study therefore appears to provide additional proof for the biological basis of the FAB classification. As suggested by Browman et al,[49] Drexler and Minowada,[27] and Neame et al,[50] and as recently reviewed by Crocker and Burnett,[51] cytochemistry and immunophenotyping greatly improve the diagnostic utility of the FAB classification of AML. With the addition of the BM-1 MoAb, further improvements in typing AML may be possible.

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Evidence That Microenvironmental Factors Account for the Age-Related Decline in Neutrophil Function

By David A. Lipschitz, Kodetthoor B. Udupa, and Laurence A. Boxer

We measured the function of neutrophils harvested from the supernatant of long-term marrow cultures in which stromal cell cultures derived from young mice were recharged with hematopoietic cells from old mice and vice versa. The functions measured were superoxide generation and enzyme secretion (lysozyme and glucuronidase), following cell activation by either phorbol myristate acetate (PMA) or Formyl-methionyl-leucyl-phenylalanine (FMLP). In addition we measured cytosolic calcium concentration and its increase following activation by FMLP. In all culture combinations recharge resulted in the recovery of $>2 \times 10^6$ cells/flask (95% neutrophils, 98% viable). Histologic studies of cytoplasmic markers indicated that recovered neutrophils were derived from the stem cell population employed for recharge. For each neutrophil parameter measured, function was markedly improved when old hematopoietic stem cells were recharged onto a young stroma and was significantly diminished when young stem cells were recharged onto an old stroma. This applied to superoxide generation, basal and stimulated enzyme levels, and to basal cytosolic calcium concentration and its increase following activation by FMLP. These results indicate that when old hematopoietic stem cells proliferate in a young microenvironment, neutrophil function returns virtually to normal. Conversely, function diminishes when young stem cells proliferate in an old stroma. These findings demonstrate, for the first time, that neutrophil function is modulated by microenvironmental factors, hormonal, cellular, or matrix, which are decreased in the elderly. That an age-related decline in function is extrinsic to the cell and is reversible has significance for the study of neutrophil function and of cellular aging and has potential therapeutic implications.

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THE LONG-TERM bone marrow culture system described by Dexter et al[1] allows sustained myelopoiesis in vitro for several months. In this system a bone marrow stromal layer provides the cellular and structural environment necessary for the proliferation of pluripotent and committed hematopoietic stem cells, their differentiation, and their maturation into neutrophils. Recently we demonstrated that neutrophils recovered from the supernatant of long-term bone marrow cultures initiated from old mice had a significant decrease in function compared to neutrophils recovered from cultures initiated from young mice.[2] This applied to measurements of respiratory burst, exocytosis, and enzyme secretion and was identical to the diminution in function of neutrophils recovered from the peritoneal cavity of aged mice.[2] Similar declines in neutrophil function have been recently reported in neutrophils obtained from healthy elderly subjects.[3-5] In this report we evaluate the role of the microenvironment in modulating the age-related decline in neutrophil function. This was done by examining the effects on neutrophil function of recharging hematopoietic stem cells derived from young or old mice onto stromal cell cultures initiated from either young or old animals.

METHODS

The approach employed was to initiate stromal cell cultures from young (4 to 6 months) or old (24 to 26 months) female C57BL/6 mice (obtained from National Institute on Aging, Bethesda, MA) utilizing the method described by Mauch et al.[6] After 4 weeks of culture the stroma was recharged with hematopoietic cells free of stromal cells obtained from young or old animals. This resulted in four combinations of cultures: young stromal-cell cultures recharged with young (young–young) or old (young–old) hematopoietic stem cells or old stromal cell cultures recharged with old (old–old) or young (old–young) hematopoietic cells At weekly intervals the supernatant and all the nonadherent cells were harvested. The medium was replaced with 6 mL of fresh medium and 4 mL of the cell-free culture medium just removed. Table 1 shows that 4 weeks after recharge greater than 2.0×10^6 cells could be recovered from the supernatant of each flask. In every case, greater than 95% of the cells recovered were neutrophils, as determined by histologic examination of cytospin preparations stained by Wright's and Giemsa reagents. Ninety-eight percent or more were viable based upon trypan blue exclusion. Cell recovery from the supernatant was less than 2×10^3 cells in nonrecharged stromal cell cultures. In addition, no growth was seen when the stem cell preparation used for recharge was added to flasks not containing stromal cell layers. We undertook preliminary studies to assure that the recovered neutrophils were derived from the stem cell population employed for recharging We used an approach developed by Reincke et al[7] that involves the use of marrow from young C57BL/6 bg/bg$_J$ ("beige") mice that are homologous with old C57BL/6 mice. The beige mice neutrophils contain giant lysosomes that can be recognized histologically on cytospin preparations stained by Sudan Black and counterstained with Giemsa.[8] Greater than 98% of recovered neutrophils contained this marker when stem cells from beige mice were recharged onto an old stromal cell culture. Conversely, when old hematopoietic stem cells were recharged onto a stromal cell culture initiated from young beige mice, none of the recovered cells contained the giant lysosomes.

Superoxide generation was assayed spectrophotometrically at 550

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Table 1. Functions of Neutrophils Recovered From Young or Old Mice Marrow Stromal Cell Cultures That Were Recharged 4 Weeks Previously With Nonadherent Marrow Cells From Young or Old Mice

Stromal cell layer derived from: Nonadherent cells obtained from.	Old Old	Young Old	Young Young	Old Young
	(Old-Old)	(Young-Old)	(Young-Young)	(Old-Young)
Cells harvested (× 10^6/flask)	2.56 ± 0 24*	3.11 ± 0.28	3.34 ± 0.24	2.59 ± 0.27
Superoxide generation (nM O_2^-/min/10^6 cells)				
Stimulated by FMLP†	0.58 ± 0 03	0.82 ± 0.02‡	0.92 ± 0.02	0.70 ± 0.02§
Stimulated by PMA	0.36 ± 0.01	0.53 ± 0.02‡	0.59 ± 0.01	0.47 ± 0.01§
Lysozyme (μg equivalent/mg protein)				
Basal	19.4 ± 1.6	31 2 ± 3.0‡	37.3 ± 2.5	26.0 ± 2.0§
Stimulated by FMLP	31.8 ± 3.7	79 2 ± 8.6‡	106.0 ± 10.0	46.4 ± 2.6§
Stimulated by PMA	32.3 ± 5 7	79 0 ± 5.2‡	105 3 ± 4 8	48.8 ± 3.9§
β-Glucuronidase (nM/min/10^6 cells)				
Basal	0 13 ± 0.01	0.17 ± 0.01‡	0 19 ± 0.01	0.15 ± 0.01§
Stimulated by FMLP	0 20 ± 0.01	0 30 ± 0.01‡	0.32 ± 0.01	0 25 ± 0.01§
Stimulated by PMA	0.23 ± 0.01	0 29 ± 0.01‡	0.31 ± 0.01	0.26 ± 0.01§

*Mean ± SEM of five separate observations.

†Stimulation by FMLP was in presence of 10 μg cytochalasin B.

‡Old hematopoietic cells on young stromal layer significantly higher than old-old combination ($P < .001$).

§Young cell on old stromal layer significantly lower than young-young combination ($P < .001$).

nm by the reduction of ferricytochrome C (type VI, Sigma Chemical Co, St Louis) using the method of Babior et al.[9] Phorbol myristate acetate (PMA) to a final concentration of 1 μg/mL or 0.1 μmol/L Formyl-methionyl-leucyl-phenylalanine (FMLP) together with 10 μg cytochalasin B were used as the stimulant of oxidative metabolism.[10] Neutrophil enzymes were measured in the supernatant obtained after degranulation induced by the addition to the cell suspension of either PMA or FMLP in presence of 10 μg/mL cytochalasin B. β-Glucuronidase activity was assayed by measuring the release of p-nitrophenyl from p-nitrophenyl β-D-glucuronide by hydrolysis.[11] The assay for lysozyme was based on the change in light absorption when a suspension of Micrococcus lysodeikticus was incubated in the presence of lysozyme.[12] As a standard, crystalline-hen egg-white lysozyme was used.

Cytosolic calcium was measured using the fluorescent dye, Quin-2, as described in earlier reports.[13] Basal values were obtained, as were levels after treatment of the cells with 0.1 μmol/L FMLP.

The results were analyzed by computer using the Statistical Analysis System (SAS Institute, Cary, NC). All the values reported are mean ± SEM. Data sets were compared using the Students' *t* test to identify significant differences; *P* values < .05 were considered to be significant.

RESULTS

We tested the ability of the neutrophils obtained from the four combinations of cultures, namely old–old, young–old, young–young, and old–young, to generate superoxide and to secrete enzymes in response to stimulation by PMA and by FMLP. For each function measured the old–old was significantly lower ($P < .001$) than the young–young (Table 1). A highly significant ($P < .001$) improvement in function was found when old hematopoietic stem cells were recharged onto a young stromal layer (young–old). This applied both to superoxide and to basal and stimulated enzyme levels, which were not dissimilar to those found in the young–young combination. In contrast, function was significantly decreased ($P < .001$) when young hematopoietic stem cells were recharged onto an old stromal cell layer (old–young).

FMLP-induced activation of the neutrophil is mediated through a calcium dependent pathway.[14] We have shown that aging is associated with a reduction in basal neutrophil cytosolic calcium concentration and a diminished mobilization to the cytosol following FMLP activation.[15] We measured basal cytosolic calcium concentration and its increase following stimulation by FMLP in neutrophils obtained from the four combinations of cultures. A basal calcium concentration ($[Ca^{2+}]i$) of 64.7 ± 1.6 nmol/L in the old–old improved significantly to 80.8 ± 1.8 nmol/L in neutrophils recovered from the young–old cultures ($P < .001$, Fig 1). Similarly a basal cytosolic calcium concentration of 73.0 ± 1.9 nmol/L in the old–young was significantly less than 94.9 ± 1.7 nmol/L in the young–young ($P < .001$). Neutrophils harvested from the young–old combination increased their cytosolic calcium concentration by 62.4% ± 4.8% following

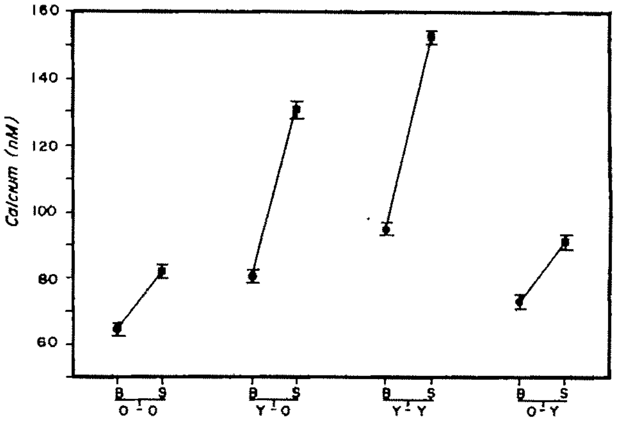

Fig 1. Cytosolic calcium concentration measured by Quin 2 fluorescence, basally (B-basal) and following activation by 0.1 μmol/L FMLP (S-stimulated) in neutrophils harvested from the cultures in which nonadherent hematopoietic cells obtained from old mice were recharged onto stromal layers derived from old (O–O) or young (Y–O) animals. In parallel, nonadherent hematopoietic cells obtained from young mice were recharged onto stromal layers initiated from young (Y–Y) or old (O–Y) animals. The values represent the mean ± SEM of five separate observations.

treatment with FMLP compared to an increase of 27.1% ± 5.1% in the old–old ($P < .001$). Mobilization was increased by 24.9% ± 4.4% when old–young neutrophils were treated with FMLP, compared to an increase of 60.3% ± 5.8% ($P < .001$) in the young–young (Fig 1).

DISCUSSION

Most studies on the aging hematopoietic system have focused on the effects of age on the pluripotent hematopoietic stem cell (CFU-S). Serial transplantation experiments have demonstrated that CFU-S gradually lose their ability to self-replicate.[16,17] In addition, the replicative potential of CFU-S derived from old donors is significantly less than CFU-S derived from young donors. However, there is evidence that extracellular factors may affect the proliferative potential of CFU-S; for example, the rate at which young CFU-S age can be accelerated by allowing them to replicate in old recipients. In addition, more recent in vivo observations have questioned the age-related decline in CFU-S and have suggested that early serial transplantation studies were related to methodologic artifact.[18-21] Even if their life span is finite, it is clear that CFU-S has a reserve capacity to produce adequate numbers of hematopoietic cells for periods that far exceed the maximum life expectancy of the animal.[22]

In recent years long-term bone marrow culture has been developed as a model to study hematopoietic aging. Evidence has been presented that CFU-S with a high replicative history are likely to be recruited into committed progenitor cells more readily than CFU-S that have divided a fewer number of times.[23] We and others have shown that the maintenance of hematopoiesis in long-term bone marrow cultures varies inversely with the age of the donor from which the culture was initiated.[6,24] Additional evidence for a finite replicative capacity for stem cells has been obtained in a series of elegant studies in which stem cell kinetics and myeloid cell production was examined in long-term bone marrow cultures subjected to varying doses of irradiation.[25,26]

We have extended the long-term bone marrow culture system to examine the effect of donor and culture age on terminally differentiated neutrophils.[2] The modest declines in neutrophil function that have been demonstrated in humans and in mice persist in neutrophils harvested from long-term bone marrow cultures initiated from old animals.[3-5,27] In this study we have examined the role of stromal variables in the age-related decline in neutrophil function. Our results clearly demonstrate that neutrophil function is modulated by a microenvironmental factor that is deficient in the aged. The role of the microenvironment and of nonhematopoietic cells in the regulation of hematopoiesis is well described. Hematopoietic cell production has been shown to be modulated and perhaps regulated by lymphocytes, macrophages and endothelial cells located within the marrow stroma.[28,29] In addition, stromal cells are capable of elaborating soluble factors that influence hematopoietic cell proliferation and/or differentiation.[30-34] There is also evidence that the composition of the extracellular matrix affects hematopoiesis in culture by a mechanism that may be organ specific.[35] Our results provide the first evidence that the marrow microenvironment affects myeloid end-cell function. Two possible stromal variables have been shown to affect the neutrophil. First, colony-stimulating factor (CSF) produced by stromal cells not only enhances myeloid cell proliferation but also stimulates neutrophil function directly.[36] It is possible that different concentrations or functions of CSF in the microenvironment of either young or old marrow may prime the neutrophil and alter its function. In other studies employing long-term bone marrow culture, however, we have shown that aging is not associated with a diminution in CSF production in day-to-day levels or in functional activity of this hormone.[37] This makes it unlikely that CSF differences in these cultures explain the effects that we have noted. The second variable worthy of consideration is the laminin–neutrophil interaction. Recently neutrophil adhesion and function have been shown to be affected by the expression of cell surface receptors that bind to laminin.[38] Different concentrations of laminin in stroma of different ages may affect receptor expression and modify cellular function.

The data provides new light on the mechanism of the age-related decline in neutrophil function. Studies in numerous cell systems have shown an age-related reduction in a series of metabolic events. The neutrophil is an ideal model to examine the effects of aging, as activation of the cell results in 100-fold or more amplification of many metabolic processes that maximize age-related differences less obvious in the basal state. Our results indicate that the demonstrated decline in neutrophil function is primarily extrinsic to the cell and correctable. The microenvironment thus appears to play a very important role in aging and now deserves serious consideration as a central factor accounting for a large number of cellular, functional, and proliferative events that accompany normal aging.

The improvement in calcium homeostasis that we describe deserves special mention. First, the increase noted in superoxide generation and enzyme release when old hematopoietic stem cells proliferate in a young microenvironment may be directly or indirectly ascribed to the improvement in calcium homeostasis noted. Second, altered calcium responses have been described in several aged-cell and organ systems, including salivary gland and heart muscle, and are thought to account for many of the reported declines in cell function that occur with aging.[39,40] We and others have obtained strong evidence that the cell membrane is very important in these changes.[15,41,42] A likely hypothesis is that the age-related decrease in neutrophil function is the result of a reversible membrane defect that is dependent primarily on the environment in which hematopoietic stem cells proliferate. The delineation and identification of the microenvironmental variables will greatly assist in defining mechanisms of both neutrophil regulatory mechanisms and of cellular aging and will have implications for the therapeutic approach to the management of elderly individuals.

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The Effect of Lithium on Growth Factor Production in Long-Term Bone Marrow Cultures

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We have previously reported that lithium chloride (LiCl) stimulates the production of granulocyte–macrophage colony-forming cells (GM-CFC), pluripotent stem cells (CFU-S), and differentiated granulocytes, macrophages and megakaryocytes in murine Dexter marrow cultures and that this effect appears to be mediated indirectly by a radioresistant adherent marrow cell. In this study we have established that exposure of murine Dexter cultures to LiCl (4 mEq/L) causes an increase of colony-forming cell megakaryocytes (CFU-meg) over 1 to 6 weeks of culture in both supernatant (188% to 611%) and stromal phases (123% to 246%). Moreover, we have shown that lithium treatment of either irradiated (1,100 rad) or unirradiated stromal cells increased production of activities stimulating formation of megakaryocyte, granulocyte, macrophage, and mixed lineage colonies and proliferation of the factor-dependent cell line, FDC-P1. This FDC-P1 stimulatory activity was completely blocked by an antibody to purified recombinant granulocyte–macrophage colony stimulating factor (rGM-CSF). The baseline or lithium-induced–stromal-derived bone marrow colony stimulating activity was partially blocked by the antibody to rGM-CSF and by an antibody to purified colony stimulating factor I (CSF-1); the two antibodies combined resulted in greater than 90% inhibition of the lithium-induced marrow stimulatory activity. In addition, radioimmunoassay (RIA) showed that although CSF-1 was detectable in supernatants of these cultures, exposure to lithium did not increase CSF-1 levels. These data indicate that Dexter stromal cells produce CSF-1 and GM-CSF and that lithium appears to exert its stimulatory effects on in vitro myelopoiesis by inducing production of GM-CSF.
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LEUKOCYTOSIS has been recognized as a side effect of lithium therapy since 1950,[1] and the use of lithium therapy for various hematopoietic disorders has been explored.[2-5] The stimulatory effect of lithium has been found mostly in the granulocyte lineage, although it appears to act on the megakaryocyte and the erythroid lineages as well [6-9]

The mechanism of action of lithium on hematopoiesis has not been elucidated.[10-17] Some reports suggest that lithium acts directly on the stem cells,[10-11] but there are data supporting the view that lithium acts indirectly via a secondary effector cell population.[12-17] In vitro studies in which adherent layers from Dexter bone marrow cultures were exposed to lithium prior to ablation of active hematopoiesis by irradiation demonstrated that these "stromal" layers had an enhanced capacity to support in vitro hematopoiesis and suggested that lithium stimulated Dexter culture hematopoiesis indirectly by an effect on a radioresistant adherent cell.[12] Studies using the in vivo diffusion chamber system have also indicated that lithium acts indirectly, inducing a humoral factor that stimulates myelopoiesis.[13] In 1980 Broxmeyer et al[14] demonstrated that lithium is capable of abrogating lactoferrin-mediated inhibition of monocyte granulocyte–macrophage colony stimulating activity (GM-CSA) production. These results together with other findings[15,16] have fostered the proposal that lithium acts via a macrophage or T cell mechanism to alter GM-CSA production. Chatelain et al[17] have suggested that lithium's action is on an accessory cell population requiring T lymphocyte function to produce stimulatory activities that then cause increased granulopoiesis and megakaryocytopoiesis. These studies indicate that lithium acts indirectly via a secondary cell population.

We have utilized liquid Dexter cultures[18] of murine marrow to elucidate the mode of action of lithium. Previous studies have established that when lithium is added to this system the number of pluripotent stem cells, granulocyte–macrophage progenitors, macrophages, granulocytes, and megakaryocytes are increased.[19] Lithium has also been shown to stimulate other hematopoietic precursors in Dexter culture: the colony-forming unit diffusion chamber (CFU-D)[20] and the high proliferative potential colony-forming cell (HPP-CFC).[21] In the present studies we have used the Dexter culture system to investigate the action of lithium on megakaryocytopoiesis. Furthermore, by using antibodies directed against various well-characterized growth factors, we have identified the cytokine responsible for the stimulatory activities of lithium in the Dexter system.

MATERIALS AND METHODS

Female C57bl/6J, BDF, (Jackson Laboratories, Bar Harbor, ME) and ICR (Dominion Laboratories, Dublin, VA) mice 8 to 14 weeks of age were used as marrow donors in these experiments

Liquid bone marrow cultures Liquid cultures were established using a modification of the method of Dexter et al,[18] as previously published.[19] Corning tissue culture flasks (25 cm^2) were inoculated with bone marrow cells in 10 mL Fischer's media with penicillin-streptomycin-fungizone, 10^{-7} mol/L hydrocortisone and 20% horse serum. Cultures were established by either flushing the marrow contents of a single tibia and femur directly into each flask or seeding each flask with 40×10^6 cells from a pooled single cell suspension. The cultures were incubated at 33°C and 5% CO_2 and refed weekly by removing half the volume from each flask and replacing it with fresh medium The nonadherent (or supernatant) cells were thus collected each week and were pooled for total cell counts and differential counts. The adherent (or stromal) cells were collected at different weekly time points by trypsinization using a 0.5% trypsin-EDTA solution (GIBCO, Grand Island, NY).[19] The reaction was stopped by adding cold Hanks' balanced salt solution

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with 5% fetal calf serum (FCS). A single-cell suspension was made and total counts and differentials carried out.

In experiments in which megakaryocytic progenitor cells were assayed, cultures were established with either 0 or 4 mEq/L lithium chloride (Sigma Chemical Co, St Louis). The same amount of lithium was added weekly in the medium used to refeed the cultures. The supernatant and stromal cells collected weekly were pooled from five to ten flasks per group and counted by hemocytometer to determine the total number of cells per flask The cells were then washed twice to remove residual lithium and assayed for colony-forming cell megakaryocytes (CFU-meg) in soft agar clonal cultures. Cytocentrifuge slide preparations were made and stained with Wright's-Giemsa stain to allow morphological identification of the differentiated megakaryocytes. A Miller disc was used and the number of megakaryocytes per 1,000 to 15,000 cells determined Some cytospin slides were stained with acetylcholinesterase, a specific marker for murine megakaryocytes,[22] and the number of positively stained cells were also determined.

Clonal agar assay. A modification of the soft agar culture technique of Williams and Jackson[23] was used to assay bone marrow progenitor cells, as described previously.[24] A supplemented McCoy's 5A media with 1×10^{-4} mol/L 2-mercaptoethanol and 15% FCS was employed for cloning the marrow cells in single layer cultures in 35-mm^2 petri dishes. When megakaryocyte precursors (CFU-meg) were assayed, the stimulus used was either pokeweed mitogen spleen-conditioned media (PWM-SCM)[24] or interleukin 3 (IL 3; courtesy of Dr James Ihle, Frederick, MD).[25] The culture dishes were incubated for seven days at 37°C and 5% CO_2, fixed with 10% formalin, and whole agar slide preparations were made.[26] The slides were then stained with acetylcholinesterase and counterstained with hematoxylin. Megakaryocyte colonies were scored as aggregates of three or more acetylcholinesterase-positive cells.

Assays for stimulatory factors induced by lithium Liquid Dexter cultures were depleted of nonadherent cells at 3 weeks of culture and refed with 10 mL fresh media, with or without 4 mEq/L lithium chloride. The stromal layers were then exposed to 0 or 1,100 rad using a Gamma cell 40 machine delivering 120 to 128 rad/min. Conditioned media (cm) was collected at various time points after the addition of lithium and irradiation: 1, 2, 4, and 7 days, and after 1 and 2 days following the seven-day refeeding of the cultures. The cm was assayed for factors stimulating bone marrow progenitor cells using the clonal agar culture system described above. Concentrated (Amicon YM10 membranes, Amicon Corp, Lexington, MA) cm was mixed directly in the McCoy's agar layer, while unconcentrated cm was added in volumes up to 1 mL on top of the pregelled agar plates. The culture dishes were incubated at 37°C, 5% for seven days.

Alternatively, bone marrow stimulatory activity was assayed using an in situ agar overlay system [26] In this technique the adherent cells in the Dexter flasks are directly overlaid with two separate layers of McCoy's nutrient media mixed with agar, with fresh marrow target cells included in the top layer. The cultures were incubated for seven days and scored for total colony growth.

The cm was also assayed for stimulatory activity using the FDC-P1 or 32D cell lines (courtesy of Dr James Ihle, Frederick, MD). Tritiated thymidine incorporation (^3H-TdR) into the cells was measured following a 24-hour exposure to the cm.[25] The cell lines were also used as the target cell population in the clonal agar assay described above, and colonies of greater than 20 cells were scored after seven days of incubation at 37°C and 5% CO_2

Lastly, the conditioned medium was assayed for colony-stimulating factor-1 (CSF-1) by radioimmunoassay (RIA; courtesy of Drs Richard Shadduck and Abdul Waheed, Pittsburgh).[27].

Antibody blocking of cm. The ability of polyclonal antibodies against recombinant murine GM-CSF or IL 3 to inhibit FDC-P1 or bone marrow colony proliferation induced by the Dexter culture cm was assessed by adding the antibodies directly to the agar cultures in Terasaki plates (FDC-P1) or in 96-well Costar plates (bone marrow). In the experiments utilizing Terasaki plates, 4 μL of McCoy's media and agar mixture containing 2,000 FD cells were added into each well, allowed to gel, and followed by 4 μL of 1x or 5x cm and 4 μL of antibody at a final dilution of 1:15 to 1:60. Normal rabbit serum at the same dilution was added as controls. Colonies of greater than 20 cells were counted after four and seven days of incubation at 37°C and 5% CO_2. For the 96-well plates, 60 μL of McCoy's media and agar mixture containing 12,000 bone marrow cells was added into each well, and followed by the same volume of 5x cm and antibody at a final dilution of 1:120. Antibodies against purified CSF-1 were also added into the bone marrow cultures at a final dilution of 1:48. The various antibodies were titered against their purified growth factors for their capacity to inhibit growth factor-stimulated cell growth. The dilutions described above completely neutralized the growth stimulatory effects of the appropriate purified growth factors, that is, purified IL 3, purified CSF-1, or purified recombinant *Escherichia coli*-derived murine GM-CSF

The antibodies against recombinant GM-CSF or IL 3 were obtained by Biogen, as previously reported.[28]

Purified CSF-1 and the antibody to purified CSF-1[29] were kindly provided by Dr Richard Shadduck (Pittsburgh).

RESULTS

Effect of lithium on Dexter culture cell recovery. The addition of 4 mEq/L lithium chloride to the long-term Dexter cultures enhanced cell number and total megakaryocytes over 6 weeks of culture (Fig 1). Megakaryocytes were determined by differential counts of Wright's-Giemsa-stained–cytospin preparations and were confirmed by counts performed on acetylcholinesterase-stained slides.

The adherent cell populations were also increased in the lithium flasks (Table 1). Total stromal cell and megakaryocyte recovery were higher in the lithium groups at weeks 3 and 6.

Effect of lithium on megakaryocyte precursors (CFU-meg). Dexter cultures containing 4 mEq/L LiCl had more megakaryocyte progenitor cells (CFU-meg >3 cells) than control cultures (Fig 2). Similar results were seen when the number of CFU-meg of over 20 cells was analyzed (data not shown).

Stromal CFU-meg (>3 cells) were also increased in the lithium flasks, as shown in Table 1. Comparable results were seen when colonies of greater than 20 cells were counted.

The supernatant and stromal cells were washed twice before plating for CFU-meg to remove residual lithium. We also carried out several experiments adding LiCl directly into the soft agar system to rule out any possible effect of lithium carry-over. LiCl was added at final concentrations of 0.0001 to 4 mEq/L into agar cultures containing plateau levels of PWM-SCM, and no augmentation of megakaryocyte, granulocyte, macrophage, or mixed lineage colony formation was seen (data not shown). A modest inhibition of CFU-meg was seen at the higher concentrations of LiCl, 0.8 to 4 mEq/L.

The induction of hematopoietic growth factors by lithium. Five separate experiments were carried out to determine whether lithium was inducing growth factor production from the Dexter cultures. The addition of lithium was delayed until week 3 of culture, when a well-established

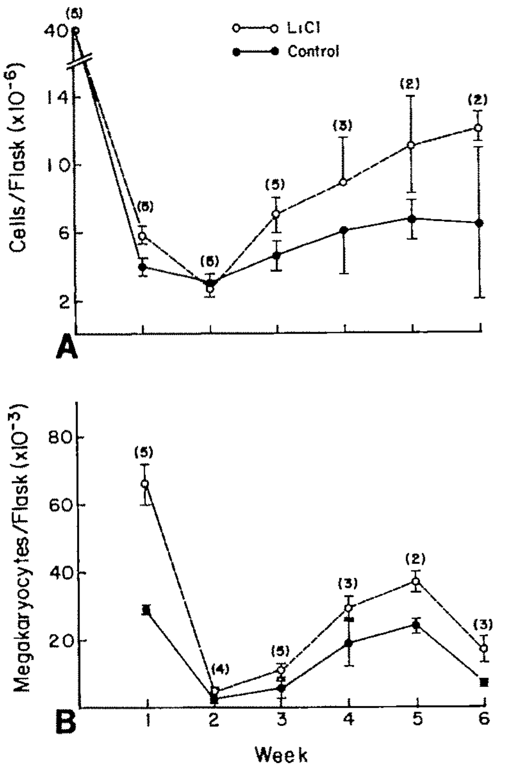

Fig 1. Weekly supernatant cell recovery from long-term marrow cultures exposed to either 0 (●—●) or 4 mEq/L (O—O) lithium chloride. Panel A shows numbers of total cells per flask over 6 weeks of culture growth. Panel B shows numbers of megakaryocytes per flask. Data are expressed as mean ± SEM of pooled suspensions from five to ten flasks per group per experiment. The number of separate experiments is shown in parentheses.

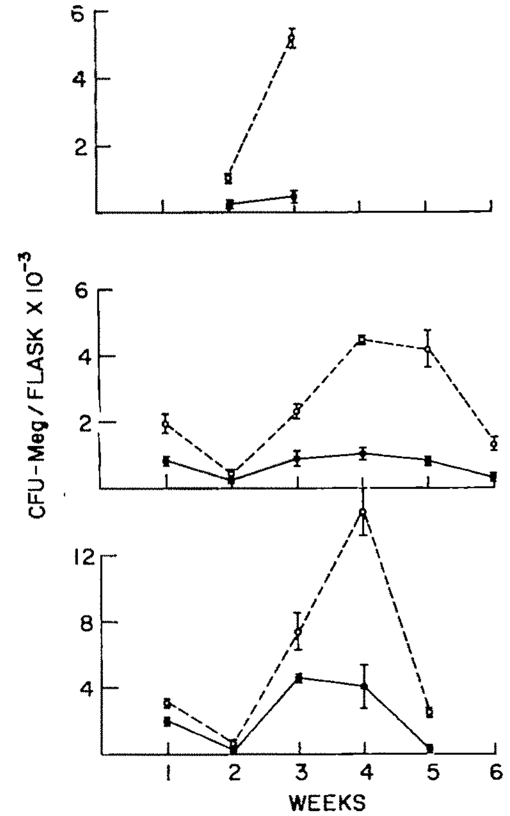

Fig 2. Weekly supernatant megakaryocyte progenitor cell (CFU-meg) recovery per Dexter culture flask following exposure to either 0 (●—●) or 4 mEq/L (O—O) lithium chloride. Data are expressed as mean ± SEM of pooled suspensions from five to ten flasks per group. Each graph represents a separate experiment.

adherent layer had formed. Lithium was then added into flasks that received 0 or 1,100 rad irradiation. When the adherent layers in the culture flasks were overlaid with bone marrow target cells, the number of colonies stimulated by the lithium flasks was significantly higher than that seen in control flasks for both the unirradiated and irradiated groups (data not shown).

Conditioned medium harvested from the flasks at several time points following irradiation and the addition of LiCl (one, two, or seven days) had increased stimulatory activity for bone marrow colony formation. Figure 3 shows the total number of colonies, or colony-forming units in culture (CFU-C), stimulated by cm harvested 24 hours after the addition of 0 or 4 mEq/L LiCl to the flasks. Panel A shows the increase in bone marrow stimulating activity in the lithium cm over control for the unirradiated groups, and Panel B shows a similar effect of lithium in the irradiated groups. The breakdown of colony types from this same experiment is shown in Figs 4 and 5. The lithium cm stimulated higher numbers of granulocyte, macrophage, mixed granulocyte–macrophage, and megakaryocyte colonies than the control cm in both the unirradiated (Fig 5) and irradiated (Fig 4) groups. CSF-1 levels were not increased by lithium treatment of either normal or irradiated stroma (Table 2). This confirms other experiments showing no effect on CSF-1 levels when 4 mEq/L LiCl was added to normal Dexter cultures out to 6 weeks of growth (data not shown).

Table 1. Stromal Cell Recovery From Long-term Dexter Cultures

Week	Group	Total Cells/ Flask × 10^{-6}	Megakaryocytes/ Flask × 10^{-3}	CFU-meg/ Flask × 10^{-3}
3	Control	7.0 ± 1	7.65 ± 3.9	3.8 ± 2.8
	Lithium	9.84 ± 0.8	39.80 ± 7.1	9.4 ± 4.3
6	Control	7.1 ± 2.8	12.10 ± 0.5	1.8 ± 0.2
	Lithium	8.0 ± 1.5	16.60 ± 6.2	2.2 ± 0.4

Week 3 total cell counts are pooled from four separate experiments, and week 3 CFU-meg counts are pooled from three experiments. Week 3 megakaryocyte counts and all week 6 counts are from two separate experiments. Data are expressed as mean ± SEM.

Lithium induction of factors stimulating FDC-P1 cells. To examine if the cm from the Dexter cultures contains GM-CSF or IL 3, the cm was assayed for activities that stimulate the FDC-P1 cell line. CM harvested from the flasks one and seven days after the addition of 4 mEq/L LiCl had increased FDC-P1 stimulatory activity whether control or irradiated stroma were evaluated and, as we have previously shown, irradiation per se increased FDC-P1 stimulatory activity. The stimulation of FDC-P1 ^{3}H-TdR incorporation by conditioned medium from lithium exposed stromal populations is shown in Fig 6. Similar results were seen in three

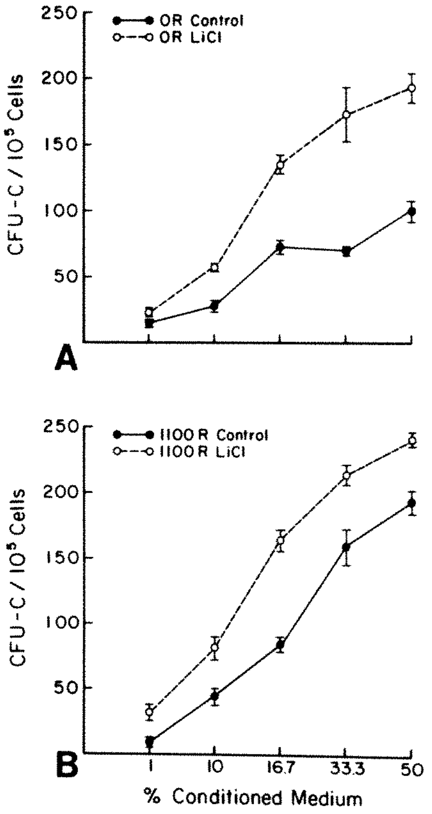

Fig 3. Assay for marrow colony-stimulating activity using conditioned medium from week 3 Dexter cultures after a 24-hour exposure to 0 (control) or 4 mEq/L lithium chloride (LiCl). (A), Dose-response curve of conditioned medium from nonirradiated (OR) cultures; (B), same dose range using conditioned medium from irradiated (1,100 rad) cultures. Colony-stimulating activity levels are expressed as mean ± SEM of total colony counts per 1 × 10^6 bone marrow target cells. These data are from one representative experiment and were confirmed using conditioned medium from four separate Dexter culture experiments.

experiments when FDC-Pl cells were stimulated by the cm to form colonies in agar.

In experiments in which a full-dose range of LiCl was added directly into the ^{3}H-TdR assay, there was some stimulation of the FDC-Pl cells by the LiCl alone at the higher dose levels (0.5 to 4 mEq/L). However, when these levels of LiCl were added to 1x cm from the control or 1,100-rad groups, there was essentially no augmentation of FDC-Pl stimulation over that seen by either cm alone (data not shown). This demonstrated that the stimulating effect of the lithium cm on the FDC-Pl cell line was not due to the LiCl acting directly on the cells.

Antibody blocking experiments. The FDC-Pl cells are known to respond to both IL 3 and GM-CSF.[28,30] Accordingly, experiments were performed assessing the effects of antibodies against each growth factor on cm-induced cell proliferation. Two clonal agar assays of the FDC-Pl cells in Terasaki plates showed 98% to 100% inhibition of colony formation by the antibody to rGM-CSF in all four groups of cm assayed (Fig 7). FDC-Pl colony growth was inhibited 0% to 9% by the antibody to rIL 3 using the lithium cm from both normal and irradiated flasks (data not shown). Previous

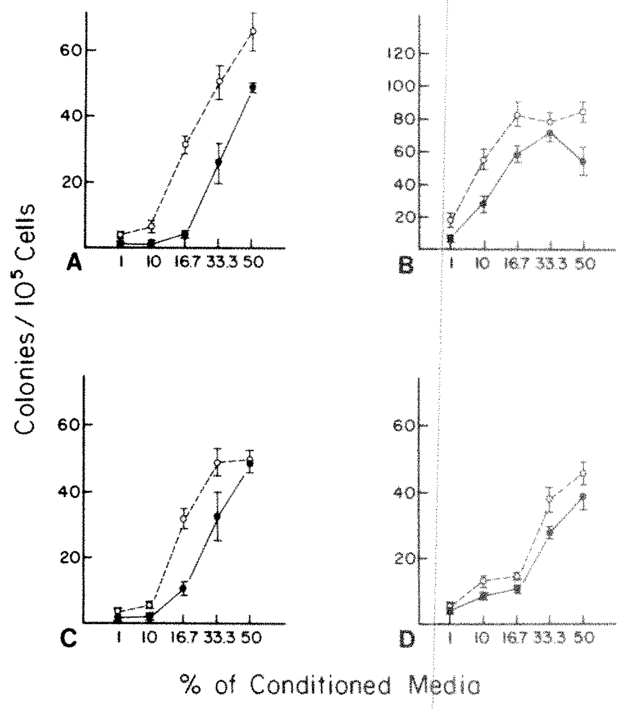

Fig 4. Morphology of colony types stimulated by irradiated culture-conditioned medium from the same experiment presented in Fig 3B. (A), Granulocyte; (B), macrophage; (C), mixed granulocyte/macrophage; (D), megakaryocyte. (●), 1,100 R control; (○), 1,100 R LiCl.

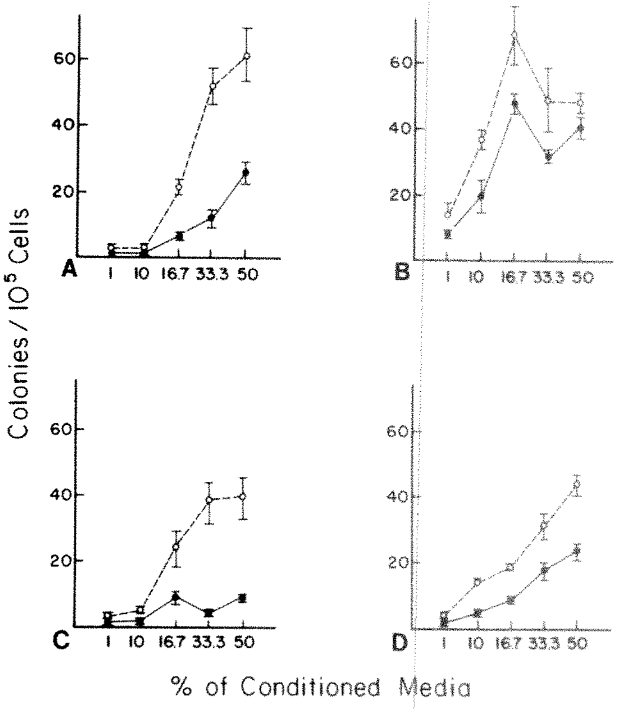

Fig 5. Morphology of colony types stimulated by nonirradiated culture-conditioned medium from the same experiment presented in Fig 3A. (A), Granulocyte; (B), macrophage; (C), mixed granulocyte/macrophage; (D), megakaryocyte. (●), OR control; (○), OR LiCl.

Table 2. CSF-1 RIA (Units/mL)

Experiment	OR C	OR Li	1,100 rad C	1,100 rad Li
1	97	129	102	106
2	51	66	74	108

Stroma were subjected to irradiation at 3 weeks of culture and exposed to LiCl (4 mEq/L) at the time of radiation. In experiment no. 1, RIAs for CSF-1 were carried out on cm collected at 24 hours (OR and OR Li) and at 48 hours (1,100 rad C and 1,100 rad Li). In experiment no. 2 cm was collected at 24 hours for all groups.

data had shown that the activity from the cm from normal or irradiated flasks (1,100 rad) was not blocked by antibodies against purified IL 3.[28] The inhibition of stromal cm stimulation of FDC-PI cells by antibody to rGM-CSF was confirmed in experiments utilizing ^{3}H-TdR incorporation into FDC-PI cells as a measure of proliferation. Further evidence that the activity stimulating the FDC-PI cells was GM-CSF was shown by the lack of stimulation by the cm of the cell line 32D, a cell line that responds to IL 3 but not to GM-CSF.

When the antibody to rGM-CSF was added to clonal agar assays with murine bone marrow cells as the target population, partial inhibition of colony growth was seen that was most prominent with the 1,100 rad Li group (Fig 8). Antibodies to CSF-1 added in this same experiment also showed partial inhibition of the marrow stimulating activity, while wells containing the combination of the two antibodies showed 71% to 99% inhibition of colony growth (Fig 8).

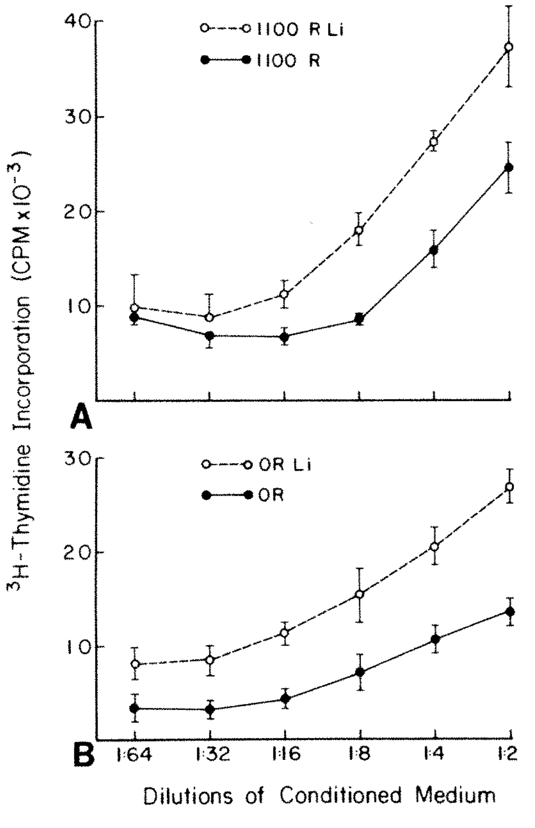

Fig 6. ^{3}H-TdR incorporation assay for FDC-PI stimulating activity in medium conditioned by Dexter adherent cells exposed for 24 hours to 0 or 4 mEq/L lithium chloride (LiCl). (A), Stimulation of FDC-PI proliferation by conditioned medium from irradiated (1,100 rad) cultures, (B), similar data for conditioned medium from nonirradiated (OR) cultures. FDC-PI cells were incubated for 24 hours with the conditioned medium, and ^{3}H-TdR was added five hours prior to termination of the cultures. The numbers are pooled from assays of conditioned medium from five individual flasks per group from one Dexter culture experiment. These data were confirmed in three separate experiments.

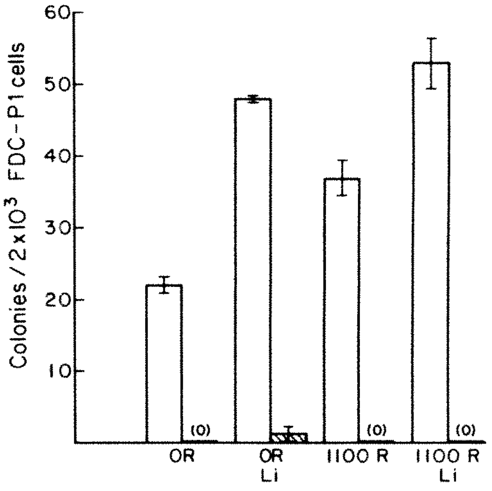

Fig 7. The effect of anti-rGM-CSF antibody on FDC-PI stimulating activity in Dexter culture-conditioned medium. Conditioned media used in this clonal agar assay was collected after 24-hour exposure of nonirradiated (OR) or irradiated (1,100 rad) cultures to 0 or 4 mEq/L lithium chloride (LiCl). FDC-PI stimulating-activity levels are expressed as mean ±1 SEM of colonies per 2 × 10^3 FDC-PI cells added in agar in Terasaki plates. These data are pooled from two separate experiments. (□), Normal rabbit sera (NRS) or (▨), antiserum containing GM-CSF antibodies (GM-CSF Ab) were added into the Terasaki wells at a final dilution of 1 : 60.

In all of the antibody blocking experiments, controls were always included that showed 100% blockage of plateau levels of purified CSF-1 by anti-CSF-1, purified recombinant GM-CSF by anti-GM-CSF, and purified IL 3 by anti-IL 3.

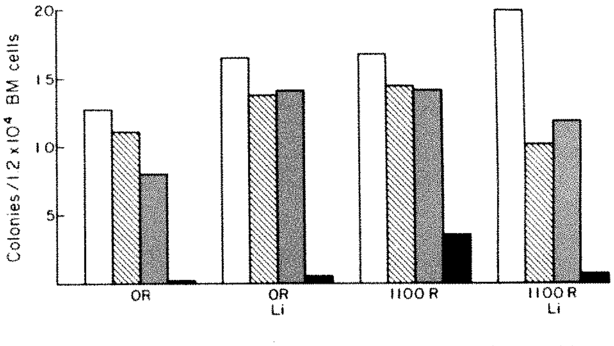

Fig 8. Inhibition of stromal-conditioned medium induced bone marrow colony formation by antibodies to rGM-CSF and purified CSF-1. Conditioned medium was collected 24 hours after Dexter adherent layers were exposed to OR or 1,100 rad irradiation and 0 or 4 mEq/L lithium chloride (LiCl). These data are from one experiment showing the mean colonies/1.2 × 10^4 bone marrow (BM) cells. NRS, normal rabbit sera; GM-CSF Ab, granulocyte-macrophage-colony-stimulating factor antibody; CSF-1 Ab, colony stimulating factor-1 antibody. □, NRS; ▨, GM-CSF Ab; ▦, CSF-1 Ab; ■, GM-CSF Ab. + CSF − 1 Ab.

There was no background stimulation of the bone marrow or FDC-Pl cells by the antibodies or normal rabbit serum alone. The antibody to rGM-CSF was selelctive in blocking rGM-CSF alone and not CSF-1 or IL 3. The antibody to CSF-1, however, did show some cross-reactivity by blocking rGM-CSF by approximately 30%.

DISCUSSION

We have previously reported stimulation of in vitro Dexter culture myelopoiesis by lithium chloride.[19] The levels of a number of differentiated progenitor cells, including CFU-S, GM-CFU-C, CFU-D, and HPP-CFC, were increased in response to LiCl.[19-21] Further data indicated that these stimulatory effects may be indirectly mediated by a radioresistant marrow adherent cell[12] and that stromal cells were sources of multilineage CSAs.[26] The present studies (Table 1 and Fig 2) show that lithium also induces increases in CFU-meg, which confirms and extends our previous observations on lithium stimulation of megakaryocyte production. Investigations by several researchers have demonstrated both in vitro and in vivo effects of lithium on a variety of hematopoietic lineages,[1-11] and a number of these studies have indicated that these effects might be mediated by influencing the production of molecules that stimulate or modulate hematopoietic stem cell differentiation.[12-17]

Our present data (Figs 7 and 8) provide evidence that lithium stimulates in vitro Dexter culture myelopoiesis by inducing secretion and/or production of GM-CSF. GM-CSF partially purified from concanavalin A (Con A)-stimulated spleen cm[24] or recombinant GM-CSF[31] can stimulate megakaryocyte, granulocyte, and macrophage differentiation. These are the three major phenotypes found in murine Dexter culture. GM-CSF also exhibits effects at the CFU-S levels,[32] and several groups have now presented evidence that GM-CSF may stimulate early erythroid stem cells.[32-34] Thus, elevations of GM-CSF could explain most of the previous reports of lithium's action in vitro and in vivo on different hematopoietic lineages[1-11]

IL 3, another growth factor having a wide range of hematopoietic bioactivity, was a prime candidate as a regulator for Dexter culture hematopoiesis. However, the present data showing no effect of anti-IL 3 antibodies on stromal-derived growth factors and no effect of stromal cm on the IL 3 responsive 32D cell line strongly indicate that IL 3 is not a regulator in this culture system. As to CSF-1, although it is produced in Dexter cultures, we have not found elevations of this factor after irradiation,[26] lectin,[28] or lithium exposure, manipulations that all appear to increase GM-CSF production in this system. This may in part be because of binding and utilization of CSF-1 by adherent macrophages, the major cellular component of the stromal cells remaining after exposure to 1,100 rad. CSF-1, however, does not stimulate megakaryocytes or FDC-Pl cells and has a relatively small effect on granulocyte production. Our experiments (Fig 8) showing virtually complete inhibition of cm-induced marrow cell proliferation by a combination of antibodies to CSF-1 and GM-CSF suggest that these two growth factors are the critical hematopoietic differentiating hormones controlling Dexter culture-differentiated cell production, but the stimulation of hematopoiesis seen in response to lithium appears due to a selective induction of GM-CSF secretion.

Lithium chloride may also induce other growth factors acting on earlier stem cells. Elevations of GM-CSF would not seem to account for the Li-induced stimulation of HPP-CFC.[21] This latter cell requires CSF-1 and one of several "synergistic" activities, including IL 3 and Hematopoietin-1.[35,36] We have isolated a cell line from Dexter-type cultures that produces CSF-1, GM-CSF, and a separate non-IL 3 synergistic activity that does not have intrinsic colony-stimulating activity but that synergizes with GM-CSF, CSF-1, and IL 3.[37-38] Whether a similar factor may be important in normal Dexter culture hematopoiesis and whether lithium may also induce production of such a factor remain open questions. Preliminary studies, however, suggest the possibility that a CSF-1-synergistic activity that is not IL 3 might also be induced by lithium in Dexter cultures.

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Posttranslational Processing of a Human Myeloid Lysosomal Protein, Myeloperoxidase

By William M. Nauseef

Myeloperoxidase (MPO) is a lysosomal enzyme present in the azurophilic granules of human neutrophils and monocytes and is important for optimal oxygen-dependent killing of microorganisms. The native molecule is a heterodimer composed of a pair of heavy–light protomers, each containing a 59-kDa and 13.5-kDa subunit. The intracellular processing during biosynthesis of MPO was examined in the human promyelocytic cell line HL-60. Endoglycosidase H and F digestion of immunoprecipitated pro-MPO demonstrated the presence of five N-linked–high-mannose oligosaccharide side chains and no complex mannose units. Incorporation of the threonine analogue β-hydroxynorvaline produced species ~2.5 kDa and ~5 kDa smaller than the fully glycosylated pro-MPO, suggesting that two of the glycans were in the asparagine-X-threonine tripeptide sequence. Processing of pro-MPO occurred very rapidly, within approximately five minutes, and was best identified using glucosidase inhibitors. The presence of such inhibitors resulted in synthesis of a 92-kDa glycoprotein rather than the usually identified 89-kDa peptide. Swainsonine, a Golgi mannosidase inhibitor, did not alter the size of the earliest synthesized protein, suggesting that pro-MPO exited the endoplasmic reticulum or *cis*-Golgi proximal to the site of mannosidases. Intracellular transport and proteolytic maturation of MPO was retarded by weak bases (NH_4Cl, chloroquine) or monensin at concentrations shown to raise intralysosomal pH. However, these agents did not qualitatively alter transport nor increase secretion. Thus, although MPO biosynthesis resembled that of other lysosomal enzymes, significant differences exist, including only limited oligosaccharide processing and intracellular transport and proteolytic maturation of pro-MPO that was only retarded by alkalinizing lysosomes without affecting the products or the fraction of pro-MPO secreted. Characterization of the determinants for targeting and of the regulatory factors in processing lysosomal enzymes in myeloid cells will provide insight into the molecular mechanisms underlying common disorders such as myeloperoxidase deficiency.

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THE SYNTHESIS of lysosomal enzymes has been the focus of extensive study in cell biology over the past 10 years.[1] In general the enzymes are synthesized as larger mol wt precursors on membrane-bound ribosomes and cotranslationally glycosylated at the amide nitrogen of selected asparagine residues. The added oligosaccharide side chains are then modified by the action of various glycohydrolases and glycotransferases as the proenzyme travels through the Golgi. In many cases the sorting of lysosomal enzymes from secretory proteins and their transport to the target lysosome is mediated by a Golgi receptor for the mannose-6-phosphate residues created in the proenzyme during processing through the Golgi.[2] Much of the pioneering work in this area was done studying cultured fibroblasts. Only limited study of the processing and sorting mechanisms for lysosomal enzymes in other cell types has been performed to date. The focus of the studies presented here is the lysosomal protein myeloperoxidase (MPO), a major constituent of the azurophilic granule of human neutrophils and monocytes. MPO is a central component of the MPO-hydrogen peroxide-halide microbicidal system of polymorphonuclear leukocytes (PMNs) and thus is an important element of host defense.[3] Previous work has examined the preliminary steps in biosynthesis of MPO in the human promyelocytic cell line HL-60.[4] The purpose of this work was to extend those previous studies by applying the principles of processing and sorting derived from studies of fibroblasts to the study of MPO. The processing and intracellular transport of MPO shares many of the features of lysosomal synthesis in fibroblasts but exhibits some important differences as well.

MATERIALS AND METHODS

Leupeptin, pepstatin A, phenylmethylsulfonylfluoride (PMSF), cetyltrimethylammonium chloride (CETAB), Triton X-100, EDTA, and mol wt markers for polyacrylamide gels were obtained from Sigma Chemical Co, St Louis. Medium for tissue culture was obtained from the Cancer Center at the University of Iowa, Iowa City, except for amino acid-depleted medium, which was prepared from the 1640-RPMI Selection Kit (GIBCO Laboratories, Grand Island, NY). Radiolabeled amino acids were purchased from Amersham (Arlington Heights, IL); endoglycosidases H and F from Boehringer Mannheim Biochemica (Indianapolis); reagents for polyacrylamide gels from International Biotechnologies, Inc (New Haven, CT); ampholines for nonequilibrium gel electrophoresis from Pharmacia Fine Chemicals (Piscataway, NJ); castanospermine and swainsonine from Calbiochem (San Diego); and deoxynojirimycin from Genzyme Corp (Boston). Formalin-fixed Staph A cells were obtained from Bethesda Research Labs (Gaithersburg, MD) and were heated to 95°C in phosphate-buffered saline (PBS) with 3% sodium dodecyl sulfate (SDS) and 10% β-mercaptoethanol three times, according to manufacturer's instructions, to minimize nonspecific binding.

Growth and maintenance of HL-60 cell lines. An early passage of the HL-60 cell line was a gift from HL Malech (National Institutes of Health, Bethesda, MD). Cells were passed twice weekly and seeded at 0.3 to 0.4 × 10^6 per mL. All experiments were performed on cells passed fewer than 25 times. Cells were maintained in suspension in RPMI 1640 supplemented with 2 mmol/L glutamine, penicillin (50 IU/mL), streptomycin (50 μg/mL), and 10% heat-inactivated fetal calf serum and grown at 37°C in 5% CO_2. Cell lines were free of mycoplasma.

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Labeling with [^{35}S]-methionine. HL-60 cells were washed with methionine-free RPMI 1640 and resuspended at 1×10^6 cells/mL in methionine-free medium supplemented with dialyzed heat-inactivated fetal calf serum (FCS). At the end of 60 minutes of methionine depletion at 37°C, 25 to 50 μCi/mL of [^{35}S]-methionine was added for 30 minutes, an interval previously shown to afford maximal labeling of pro-MPO.[4] Labeling was terminated either by collecting cells and supernatant, as in pulse experiments, or by adding 10 mmol/L unlabeled methionine, as in chase experiments. At the end of the pulse or chase period, cells were transferred to ice and recovered by centrifugation at 400 g for 10 minutes. Supernatants were saved at −80°C until study and cell pellets were solubilized in 1% Triton X-100 (Tx-100) in Tris buffered saline (TBS: 10 mmol/L Tris HCl, 150 mmol/L NaCl, pH 7.4) with 5% cetyltrimethylammonium bromide, 1 mmol/L phenylmethylsulfonyl fluoride, 100 μg/mL leupeptin, 100 μg/mL pepstatin, and 1 mmol/L EDTA. The cells were vortexed vigorously and held in a freezing ice bath for 30 minutes. Insoluble material was removed by ultracentrifugation (180,000 g × 10 minutes; Airfuge, Beckman Instruments) and the cell extract stored at −80°C until analysis.

Labeling with [^{3}H]-threonine. HL-60 cells were suspended in threonine-free (THR-free) RPMI 1640 with 10% dialyzed heat-inactivated FCS. Cells were depleted of THR for 60 minutes and then labeled with 10 μCi/mL of [^{3}H]-THR for varied time intervals. Labeling was terminated as described in pulse experiments above.

Analysis of labeled proteins. Immunoprecipitations were carried out in two steps as described previously.[4] An aliquot was quantitated by scintillation spectroscopy and the remainder analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography.

Inhibition of glycosylation with β-hydroxynorvaline. The THR analogue β-hydroxynorvaline (Hnv)[5-7] was dissolved in sterile methionine-free, THR-free RPMI 1640 at 50 mmol/L. Cells were suspended in supplemented medium with Hnv with or without an excess (100 mmol/L) of THR for four hours prior to labeling.

Inhibitors of glycoprotein processing. Stock solutions of castanospermine (10.58 mmol/L), deoxynojirimycin (100 mmol/L), and swainsonine (5.8 mmol/L) were prepared sterilely using methionine-free RPMI 1640. Cells were incubated for one hour in varied concentrations of the inhibitors prior to pulse/pulse-chase experiments carried out with inhibitors still present in the medium.

Mannosidase activity. The activity of Golgi mannosidase II in HL-60 cells was assessed spectrophotometrically by quantitating the amount of p-nitrophenyl released from p-nitrophenyl-α-D-mannoside at pH 5.5, as described by Tulsiani et al[7] and Tulsiani et al.[16] One unit of mannosidase activity is necessary to generate 1 μmol p-nitrophenol per min.

Modification of intralysosomal pH. Cells were grown in the presence of a weak base (NH_4Cl or chloroquine) or the carboxylic ionophore monensin[8] prior to pulse-chase labeling. The pH of the stock solutions of NH_4Cl, chloroquine, and monensin were adjusted such that addition to the cell culture did not alter the pH of the medium by more than 0.1 pH unit. The effect of the agents on intralysosomal pH was determined fluorometrically using the weakly basic fluorescent probe 9-aminoacridine (9-AA). 9-AA was prepared as a stock solution of 10 mmol/L in ethanol and stored at 4°C. In assays the probe was diluted to 50 nmol/L in Hanks' balanced salt solution for measurements. Fluorescence was measured in a Perkin-Elmer spectrofluorometer (Model LS-5, Perkin-Elmer Corp, Norwalk, CT) with slit widths of 5 nm and excitation wavelength of 400 nm and an emission wavelength of 456 nm, as described previously.[9] A 3-mL reaction volume was used and stirred continuously at 37°C during fluorescence measurement. The fluorescence of 50 nmol/L 9-AA in Hanks' balanced salt solution was measured, and HL-60 cells (range of 1 to 6×10^6 cells/mL) were added, after which fluorescence was measured continuously until a steady state was reached (one to three minutes). After the steady state was reached, various amounts of NH_4Cl (0 to 100 mmol/L), chloroquine (0 to 100 μmol/L), or monensin (0 to 1.0 μmol/L) were added. In general, the new steady state baseline was reached within 30 to 45 seconds after adding the weak base or monensin. The fluorescence value at the new steady state was recorded and the solution made 0.1% Tx-100 to release all intracellular 9-AA. All values were corrected for the dilutional effects, and none of the agents added affected baseline 9-AA fluorescence at the concentrations used. Data are expressed as the percent of reversal of fluorescence quenching.

Digestion with endoglycosidases H and F. For digestion with endoglycosidase H (endo H) MPO-related peptides were immunoprecipitated in the usual fashion and 50 μL of 2% SDS were added to the Cowan cell antigen–antibody complex prior to heating in a boiling water bath for two minutes. The microfuge tube was then placed on ice for 30 minutes, after which the protein A and insoluble SDS were centrifuged out at 4°C. The supernatant was transferred to a fresh microfuge tube to which were added 30 μL of citrate buffer (150 mmol/L sodium citrate, pH 6.0) and 5 μL 100 mmol/L PMSF. The desired amount of endo H was then added and the reaction incubated at 37°C for various periods of time. The digestion was terminated by making the reaction mixture 2.3% SDS and 5% β-mercaptoethanol and heating for two minutes in a boiling water bath. Digestion with endoglycosidase F (endo F) was performed in a similar fashion except the incubation buffer was 100 mmol/L sodium phosphate (pH 6.5), 50 mmol/L EDTA, 0.5% NP-40, 0.2% SDS, and 1% β-mercaptoethanol, as described by Elder et al.[10] The results of digestion with endo H or endo F were examined by PAGE and autoradiography.

Analytic electrophoreses. Nonequilibrium pH gel electrophoresis (NEPHGE) was performed according to the method described by O'Farrell et al.[11] Samples were prepared by incubating the Cowan cell-antigen–antibody in gel lysis buffer for at least four hours at 4°C prior to removing the Cowan cells by centrifugation. Electrophoresis was performed at a constant voltage for 1,200 volt-hours. The tube gel was soaked for 45 minutes at room temperature in SDS gel solubilizing buffer with bromophenol blue and then placed atop a 10% acrylamide slab gel and electrophoresed as described below.

SDS-PAGE was performed as previously described.[4] Resultant gels were stained for protein with R-250 Coomassie blue in 50% methanol and 10% acetic acid and destained in 15% methanol/10% acetic acid. For fluorography the gel was soaked in 20 vol water followed by 10 vol 1 mol/L salicylate (pH 5.7) for 30 minutes as described by Chamberlain.[12] Gels were dried and exposed to preflashed X-Omatic XAR-5 film (Kodak, Rochester, NY). Mol wts were calculated using standards: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa).

RESULTS

Number of N-linked glycans in pro-MPO. To determine the number of N-linked oligosaccharide side chains in pro-MPO, stepwise digestion of pro-MPO with endoglycosidases was performed. Susceptibility of pro-MPO to digestion with endo H (16 hours with one unit endo H) has previously been shown[4] and indicates that pro-MPO is rich in high-mannose oligosaccharides. Immunoprecipitates of pulse experiments were treated with various concentrations of endo H or endo F over a wide range of time, demonstrating in the end that the high-mannose side chains in pro-MPO were readily cleaved by endo H (Fig 1 A) and endo F (Fig 1 B). Terminal

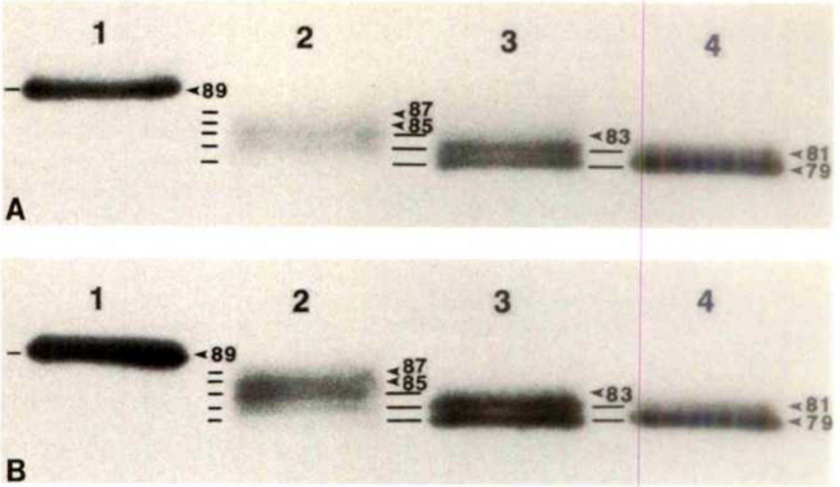

Fig 1. Endoglycosidase treatment of immunoprecipitates. Immunoprecipitates of HL-60 cells labeled for 30 minutes were digested with endoglycosidase H (A) or endoglycosidase F (B) prior to SDS-PAGE and autoradiography. Sequential digestion either by endo H with 5 mIU (lane 2), 10 mIU (lane 3), or 15 mIU (lane 4) for ten minutes at 37°C or by endo F with 20 mIU for one minute (lane 2), three minutes (lane 3), or five minutes (lane 4) resulted in visualization of six "ladder" steps, consistent with the presence of five N-linked oligosaccharide side chains on the fully glycosylated pro-MPO. In addition to the fully glycosylated 89-kDa and the deglycosylated 79-kDa species, there were four intermediate products.

digestion with either endo H or endo F produced a 79-kDa species of the same mol wt, suggesting that pro-MPO contained only high-mannose side chains and lacked complex mannose groups. Furthermore, there were four intermediate products of partial digestion with either of the endoglycosidases (Fig 1). These species migrated with mol wts of 87 kDa, 85 kDa, 83 kDa, and 81 kDa. Therefore the 89-kDa pro-MPO contained five high-mannose oligosaccharide side chains and no complex mannose groups.

Inhibition of THR incorporation by Hnv. To assess the pattern of N-linked glycosylation we used the THR analogue Hnv.[5-7] Incorporation of Hnv into a protein changes the tripeptide sequence ASN-X-THR to ASN-X-Hnv, thereby destroying those glycosylation sites normally in the ASN-X-THR sequence. HL-60 cells were suspended in THR-deficient medium for 16 hours with no decrease in cell viability, as judged by exclusion of trypan blue. [^{3}H]-THR was added and the cells labeled for 4 hours. Cells incorporated 38.9 pmol of THR per 10^6 cells. In the presence of Hnv, THR incorporation was inhibited in a dose-dependent fashion (Table 1). Labeling in the presence of 2.0 mmol/L Hnv resulted in reduction in THR incorporation to 28% of the control value. When cells were labeled with [^{35}S]-methionine in the presence of 2.0 mmol/L Hnv and the absence of THR, the amount of MPO that was immunoprecipitated was 49.7% of control.

To identify the effects of Hnv on the synthesis and fate of pro-MPO, cells were incubated with 2.0 mmol/L Hnv for four hours in THR-free medium or in the same medium to which an excess of unlabeled THR was added. Cells were then resuspended in medium free of THR and methionine with added Hnv in the presence or absence of an excess of unlabeled THR for one hour. Cells were then labeled for 30 minutes with [^{35}S]-methionine, an interval of time for optimal labeling of pro-MPO.[4] Under these conditions the control produced normal pro-MPO, a single 89-kDa glycoprotein[4] (Fig 2, lane a). Cells that incorporated Hnv produced two species that migrated at ~86.5 kDa and ~84 kDa, respectively, rather than at the normal 89 kDa (Fig 2, lane b). When cells were chased for 17 hours, during which time normal pro-MPO is processed into the 59-kDa and 13.5-kDa

Table 1. Effects of β-Hydroxynorvaline on Threonine Incorporation

Hnv	THR-Incorporation Into Protein (%)	MPO Synthesized (%)
0.0	100.0	100.0
0.5 mmol/L	88.6	100.0
1.0 mmol/L	69.4	100.0
2.0 mmol/L	28.6	49.7
3.0 mmol/L	15.6	37.3

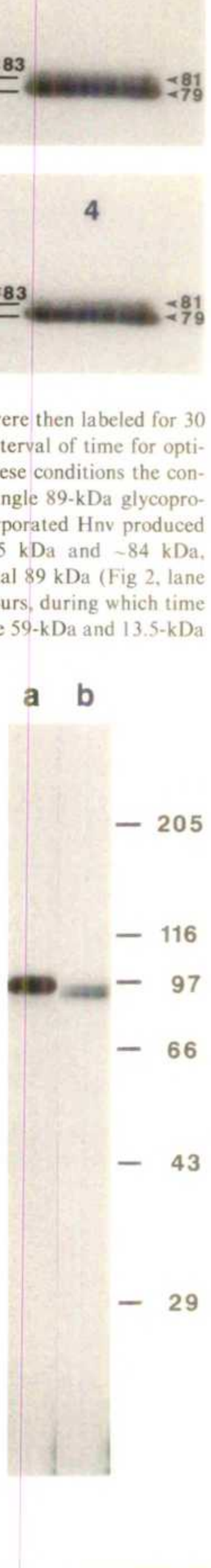

Fig 2. Effects of threonine analogue, β-hydroxynorvaline, on MPO synthesis. HL-60 cells were incubated in the presence of 2.0 mmol/L Hnv with (lane a) or without (lane b) excess threonine prior to labeling for 30 minutes with [^{35}S]-methionine. Under control conditions (lane a) an 89-kDa species was immunoprecipitated with rabbit antiserum to MPO. Without added threonine (lane b), incorporation of Hnv into the nascent protein resulted in immunoprecipitation of two proteins of 86.5 kDa and 84 kDa.

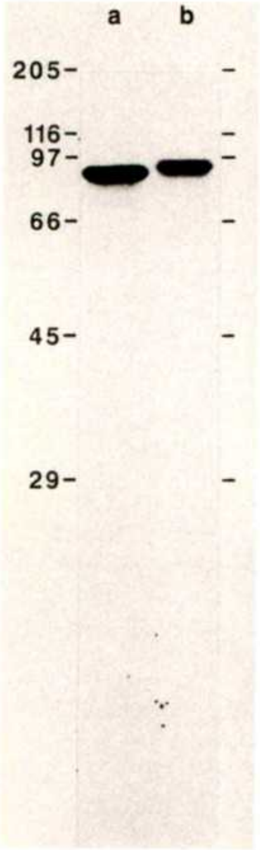

Fig 3. Effect of castanospermine on MPO biosynthesis. Immunoprecipitates of HL-60 cells labeled in the absence (lane a) or presence (lane b) of 2.5 mmol/L castanospermine were separated by SDS-PAGE followed by fluorography. Under control conditions (lane a), an 89-kDa protein was identified. Treatment with the glucosidase inhibitor castanospermine resulted in synthesis of a 92-kDa species.

subunits of mature MPO, these less glycosylated species were neither processed into smaller peptides nor secreted into the culture medium, suggesting that modification of the glycosylation of pro-MPO significantly altered processing. The effects of Hnv substitution on the charge of pro-MPO were examined by analysis of immunoprecipitates by two-dimensional electrophoresis (NEPHGE × SDS-PAGE) followed by autoradiography (data not shown). Because of the conditions under which the NEPHGE is run and the cationic nature of MPO, true isoelectric points are not measured. However, after 1,200 volt-hours, the 89-kDa pro-MPO as well as species produced in the presence of Hnv had migrated to approximately 7.36 (7.28 to 7.44).

Role of glycoprotein processing. The events in processing of the N-linked oligosaccharide side chains were examined by assessing the effects that various inhibitors of specific enzymes in the processing pathway had on the product. Immunoprecipitates from cells labeled and chased in the presence of 2.5 mmol/L castanospermine, an inhibitor of glucosidase I,[13] were examined by SDS-PAGE (Fig 3). As shown, in the presence of castanospermine, the immunoprecipitated pro-MPO migrated with an apparent mol wt of 92 kDa, rather than the previously identified 89 kDa. Identical results were seen when labeling was done in the presence of 1 mmol/L deoxynojirimycin, an inhibitor of glucosidases I and II[14] (Fig 4). As the concentration of deoxynojirimycin was increased to 0.5 mmol/L, 1.0 mmol/L, and 2.0 mmol/L, less 92-kDa peptide was deglycosylated to an 89-kDa peptide. Another α-glucosidase inhibitor, bromoconduritol,[15] had the same effect on pro-MPO processing (data not shown). In the presence of any of these glucosidase inhibitors, the 92-kDa protein was processed into normal MPO subunits, but the rate of processing was slower than normal, and there was a slight decrement in the amount of pro-MPO secreted.

In contrast to the effects of inhibitors of enzymes that are located in the proximal to the *cis*-Golgi, swainsonine, an inhibitor of mannosidase II,[16] had no effect on the processing or secretion of pro-MPO, even when used in concentrations as high as 24 μmol/L (Fig 5). As shown in Table 2, this concentration of swainsonine reduced the mannosidase activity in HL-60 cells to 8.1% of the activity of control cells. When HL-60 cells were pulsed and chased for 17 hours in the presence of swainsonine, normal processing into mature MPO occurred. Since Golgi α-mannosidase II is located in the medial cisternae of the Golgi,[1] whereas the glucosidases are in the endoplasmic reticulum, pro-MPO appeared to be transported into a prelysosomal compartment very early and not to undergo extensive processing after the initial actions of the glucosidases.

Effects of weak bases on intralysosomal pH. Before using agents to explore the importance of intralysosomal pH on intracellular processing of pro-MPO, the effects of these agents on lysosomal pH was quantitated using the weakly basic fluorescent probe 9-AA. As previously described,[9] 9-AA accumulates in acidic intracellular compartments such as lysosomes and exhibits a concentration-dependent quenching of fluorescence such that a decrease in 9-AA concentration results in a reversal of fluorescence quenching that can be measured spectrophotometrically. The fluorescence of 9-AA is itself unaffected by changes in pH.

The ability of various weak bases to increase intralysosomal pH was monitored by the reversal of quenching of 9-AA fluorescence. The quenching of fluorescence was dose-dependent for each of the agents examined: there was approximately 50% reversal at 10 mmol/L NH_4Cl, 75 μmol/L chloroquine, and 0.25 μmol/L monensin.

Effects of weak bases on MPO processing. Pulse-chase labeling of HL-60 cells was performed in the presence of

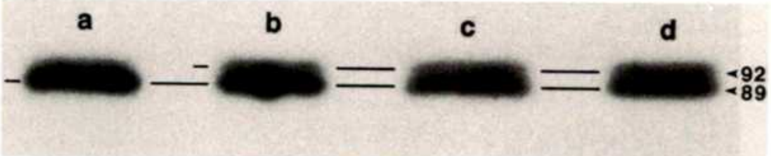

Fig 4. Effects of deoxynojirimycin on MPO biosynthesis. Immunoprecipitates of HL-60 cells labeled in the presence of 0 (lane a), 0.5 mmol/L (lane b), 1.0 mmol/L (lane c), or 2.0 mmol/L (lane d) deoxynojirimycin. With higher concentrations of deoxynojirimycin there was recovery of relatively less 89 kDa and more of the 92 kDa species, consistent with a dose-dependent inhibition of a processing step.

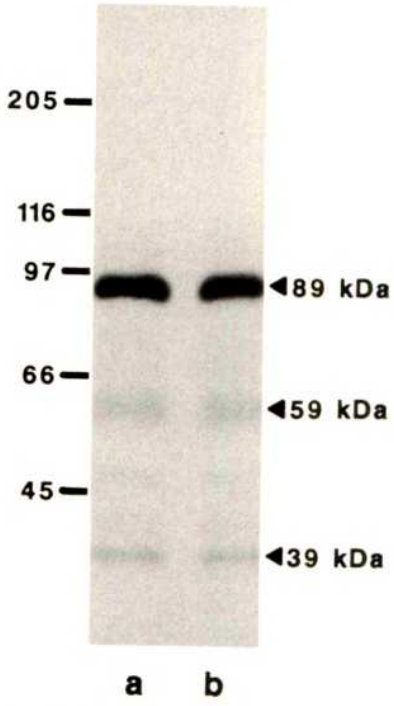

Fig 5. Effects of swainsonine on MPO biosynthesis. Immunoprecipitates of HL-60 cells labeled in the absence (lane a) or presence (lane b) of 24 μmol/L swainsonine were separated by SDS-PAGE and the gel subjected to fluorography. Cells were pulsed for 30 minutes and chased for 17 hours after the addition of unlabeled methionine. Processing of MPO was identical for both control cells (lane a) and those pulse-chased in the presence of swainsonine (lane b). The inhibition of Golgi α-mannosidase II by swainsonine did not alter the processing of MPO.

Table 2. Effects of Swainsonine on Mannosidase Activity

Swainsonine	mU mannosidase/10^8 Cells
0	0.450
6 μmol/L	0.067
24 μmol/L	0.037

weak bases at concentrations shown to raise intralysosomal pH to determine the importance of intralysosomal pH in intracellular transport and processing of pro-MPO. Cells were labeled in the presence of 10 mmol/L and 50 mmol/L NH_4Cl, concentrations that resulted in 53.3% and 74.2% reversal of fluorescence quenching, respectively. Samples were taken at the end of the labeling period (zero chase) and at 12, 24, and 44 hours after the additon of unlabeled methionine (Fig 6). Cells were viable at the end of the chase period, as judged by exclusion of trypan blue. In control cells (Fig 6, lanes 1, 4, 7, and 10), the 89-kDa pro-MPO was processed to 59-kDa, 39-kDa, and 13.5-kDa peptides, as previously described.[4] In contrast, the NH_4Cl-treated cells processed pro-MPO to the normal MPO subunits but at a much slower rate than did control cells. For example, cells grown in 50 mmol/L NH_4Cl (Fig 6, lanes 3, 6, 9, and 12) lacked the 59-kDa subunit as late as 12 hours of chase. Analysis of MPO peptides released into the medium was performed in an identical manner (data not shown) and also demonstrated that events were delayed in the NH_4Cl-treated cells, that is, whereas control cells secreted small amounts of 89-kDa pro-MPO at 12 hours, the NH_4Cl-treated cells lagged 12 hours behind, the first MPO peptides detected in the medium at 24 hours of chase.

These findings were not unique to NH_4Cl-treated cells. Pulse-chase experiments in the presence of 25 μmol/L chloroquine or 0.75 μmol/L monensin (data not shown) produced identical results. In both cases pro-MPO was

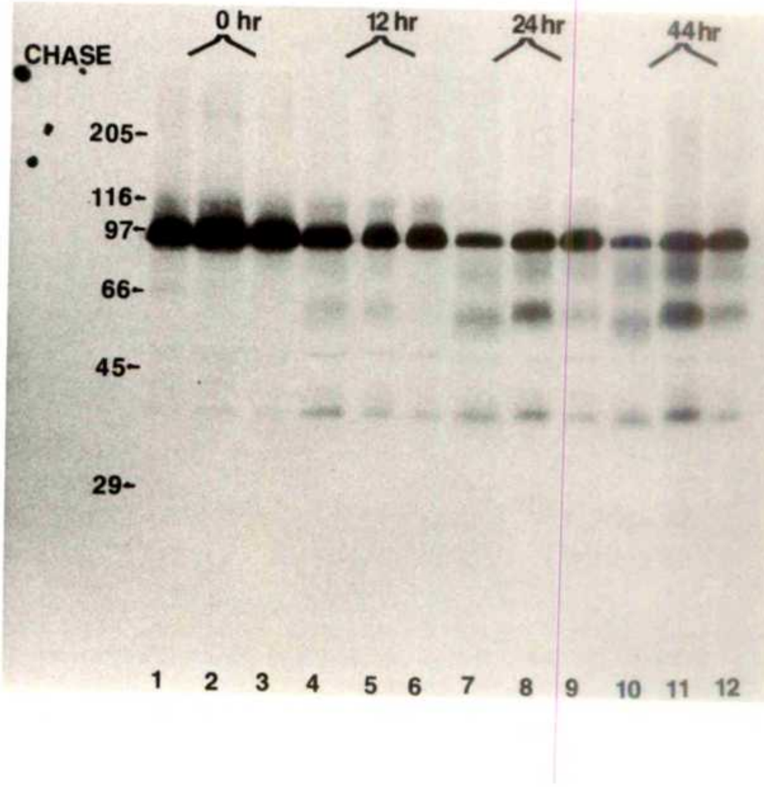

Fig 6. Pulse-chase labeling in the presence of NH_4Cl. Immunoprecipitates of HL-60 cells labeled for 30 minutes with [^{35}S]-methionine and chased for 0 (lanes 1 to 3), 12 (lanes 4 to 6), 24 (lanes 7 to 9), and 44 hours (lanes 10 to 12). Experiments were performed in the absence (lanes 1, 4, 7, 10) or presence of 10 mmol/L (lanes 2, 5, 8, 11), and 50 mmol/L (lanes 3,6,9,12) NH_4Cl. Cells labeled in the presence of NH_4Cl processed pro-MPO at a slower rate than did control cells.

processed in a qualitatively normal but markedly retarded fashion. Like control cells, treated cells secreted small amounts of pro-MPO into the medium, but the extracellular release was delayed in cells treated with either agent.

DISCUSSION

MPO constitutes 4% of the human PMN by weight and is a component of the potent MPO-H_2O_2-halide microbicidal system critical for normal host defense.[3] Despite detailed characterization of the structure and function of this important myeloid enzyme,[17] studies of its biosynthesis, processing, and intracellular transport have been limited.[4,18-22] In addition, immunochemical studies of normal and MPO-deficient PMNs, using a monospecific polyclonal antiserum to MPO, demonstrated the presence of an 89-kDa protein in addition to the normal MPO subunits. Based on that observation, we hypothesized that MPO-deficient cells synthesize a proenzyme form of MPO but that the precursor was not processed normally, resulting in the absence of functional and mature MPO subunits and the presence of an inactive pro-MPO.[23] Before testing this hypothesis directly, processing and intracellular transport of MPO during its synthesis in myeloid cells needed further characterization.

Based on studies in nonmyeloid cell systems, the synthesis and transport of lysosomal enzymes can be separated into four well-defined phases. First, there is cotranslational glycosylation of the nascent peptide chain at certain asparagine residues that appear in the tripeptide sequence ASN-X-SER or ASN-X-THR, where X is any amino acid except possibly proline and aspartic acid.[24] Second, these high-mannose side chains formed in the rough endoplasmic reticulum are processed in the Golgi; glucose and mannose groups are removed, mannose-6-phosphate residues formed, and the side chains converted to complex or hybrid structures. Third, the bulk of the enzyme is directed to the lysosome, while a minor fraction enters the constitutive pathway of secretion and is released into the culture medium. Finally, the proenzyme is proteolytically processed into mature enzyme before or after transport to the lysosome, the latter events often being pH dependent.[25]

MPO is synthesized as a precursor of higher mol wt than the mature enzyme and is slowly processed into MPO subunits.[4,18-22] The primary translation product of 80 kDa is sufficient size to account for both heavy and light subunits, suggesting the protomers are synthesized as one molecule and therefore under control of one gene. We have isolated cDNA clones for human MPO from an HL-60 cDNA library and have shown that a single gene encodes both subunits.[18] The terminal product of endo F digestion was 79 kDa, ∼1 kDa smaller than the primary translation product. This difference in mol wt between the primary translation product and the deglycosylated product of in vivo synthesis is consistent with the presence of a signal peptide that is cotranslationally cleaved. In addition, full-length cDNAs for MPO sequenced by two groups independently[26,27] contain N-terminal sequences with features of a signal peptide: the sequence is rich in nonpolar amino acids (66%), lacks glycosylation sites, and contains a potential cleavage sequence ALA-THR bond.[28]

The nascent protein then undergoes N-glycosylation. Based on the "ladder steps" seen after endoglycosidase digestion, five N-linked oligosaccharide side chains were added cotranslationally or posttranslationally. Each unit of $GlcNAc_2Man_9Glu_3$ has a molecular mass of approximately 2,620, thus one would predict that endo F digestion would reduce the mol wt of pro-MPO by approximately 11,950, since one GlcNAc molecule (molecular mass 230) would remain at each previously glycosylated asparagine. Since the endo F digestion produced a final product of 79 kDa, the fully glycosylated pro-MPO would have an estimated molecular mass of 91 kDa, in close agreement with the results obtained. The fully glycosylated pro-MPO was approximately 92 kDa, the species synthesized in the presence of castanospermine, which is rapidly processed to produce an 89 kDa species. Atkinson and Lee have previously shown that the action of glucosidase I may be so rapid as to act before polypeptide synthesis is completed.[30] As expected, the loss of three glucose molecules per oligosaccharide side chain decreased the molecular mass of the recovered protein by approximately 2,700 (5 × 540).

Two of these high-mannose side chains were added to asparagine side chains within a threonine-containing tripeptide, since inhibition by Hnv decreased the mol wt of pro-MPO by approximately 2.5 kDa and 5 kDa. Presumably the other three glycosylation sites occur in a serine-containing acceptor sequence. The smaller species were not processed in pulse-chase experiments, suggesting that such a change in the glycoprotein results in faulty transport, intrinsic instability of the species intracellularly, miscleavage of subunits, or that the enzyme responsible for proteolytic processing contains a critical threonine and substitution with Hnv results in an inactive enzyme.

The effects of a variety of inhibitors of the processing pathway present in Golgi were examined to identify intermediate species generated during maturation of pro-MPO. Three inhibitors of glucosidases, castanospermine, deoxynojirimycin, and bromoconduritol, resulted in immunoprecipitation of larger species of pro-MPO. Inhibition of deglycosylation retarded but did not inhibit proteolytic processing of MPO, similar to the accumulation of cathepsin D and β-hexosaminidase in the endoplasmic reticulum of fibroblasts treated with 20 nmol/L deoxynojirimycin.[29]

The failure of swainsonine to inhibit pro-MPO processing indicates that pro-MPO exited proximal to the site of Golgi mannosidase II, which is localized in the medial cisternae of Golgi.[1] Mannosidases present in the endoplasmic reticulum are reported to be resistant to inhibition by swainsonine, whereas those in Golgi or lysosomes are inhibited at 2 μmol/L.[34] Digestion of immunoprecipitates with either endo H or endo F resulted in a 79-kDa protein, indicating that complex- or hybrid-mannose side chains were not formed, consistent with the conclusion from the swainsonine experiment that mannosidases do not modify pro-MPO during its intracellular processing. Previous studies using endo H treatment of immunoprecipitates after >60 hours of chase demonstrated conservation of some of the high-mannose side chains in the heavy chain of mature MPO.[4] Thus, glycosylated and partially processed pro-MPO exits from the ER

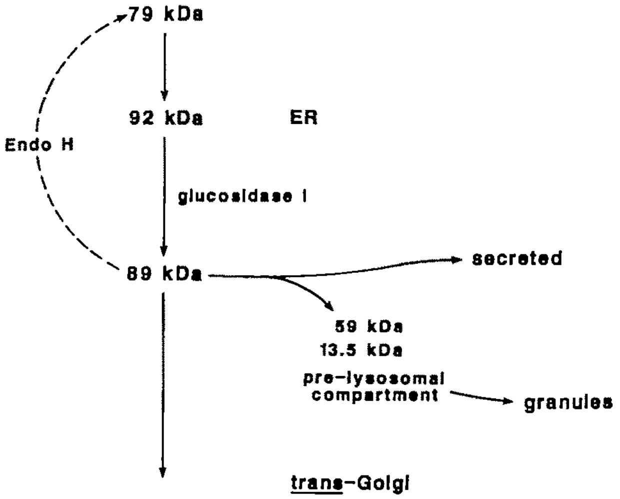

Fig 7. Model of MPO processing. Based on the data presented, pro-MPO is a 92-kDa glycoprotein produced by glycosylation of a 79-kDa peptide chain with five N-linked oligosaccharide side chains. The product of endoglycosidase digestion (79 kDa) and the primary translation product (80 kDa) differ by ~1 kDa, consistent with the existence of a signal peptide. Pro-MPO is rapidly processed to an 89-kDa glycoprotein that is stable and exits the ER or *cis*-Golgi prior to the site of any Golgi mannosidases. A small fraction of the 89-kDa species is secreted. Proteolytic maturation and eventual transport to lysosomes occurs in one or more prelysosomal compartments by a mechanism retarded but not totally inhibited by lysosomal alkalinization.

or early in the *cis*-Golgi some time after the action of glucosidase II (Fig 7). In studies of subcellular fractions of myeloid cells, only fully processed MPO has been found in lysosomes, whereas both processed and precursor forms were found in Golgi-rich fractions,[21,32] consistent with the data presented here.

Presumably pro-MPO enters a prelysosomal compartment or compartments wherein proteolytic maturation occurs. This maturation may occur stepwise, starting with proteolysis to a 74-kDa peptide, as suggested by Akin and Kinkade,[35] followed by cleavage into the heavy and light subunits of mature MPO. Our studies, however, do not address this point. In many cell systems previously described, the proteolytic processing and intracellular transport are pH dependent,[25,33] and raising intralysosomal pH inhibits this processing and shunts the uncleaved precursor form out of the cell.[25,31] These events in the biosynthesis of MPO appear to be independent of pH. Three different alkalinizing agents were used in concentrations shown to raise intralysosomal pH. Although all retarded MPO biosynthesis, none qualitatively altered the proteolytic processing, intracellular transport, or secretion of MPO. Thus, it appears that the proteolytic processing and the intracellular transport of mature MPO to azurophilic granules occurs in a prelysosomal compartment(s) by a process that is not strictly pH sensitive. Studies by Hasilik et al[20] and Stromberg et al[21] also suggest that processing of MPO is not pH dependent. Akin and Kinkade[35] have recently reported that the conversion of an MPO precursor to mature MPO occurs in isolated subcellular organelles at a neutral pH and is not inhibited by lysosomotropic agents or protonophores. Hasilik et al[20] have also shown that NH_4Cl influenced segregation and maturation of cathepsin D much less in HL-60 cells than in fibroblasts, indicating that HL-60 cells may use a mechanism for sorting of lysosomal proteins that is independent of mannose-6-phosphate residues and independent of pH. Fibroblasts from patients with I-cell disease are unable to synthesize mannose-6-phosphate residues and therefore have inadequate intracellular stores of lysosomal enzymes.[36] However, there are variable amounts of enzymes such as α-glucosidase[37] and cathepsin D[20] present in these fibroblasts, indicating an alternative pathway for intracellular transport of lysosomal enzymes must exist. In addition, many lysosomal enzymes are processed and transported normally in other cells in patients with I-cell disease,[38,39] providing further evidence that mechanisms independent of mannose-6-phosphate receptors must be regulating normal intracellular transport for certain lysosomal enzymes.

In summary, the biosynthesis and intracellular transport of MPO resembles lysosomal enzyme synthesis in other cell systems in many ways. MPO is synthesized as a precursor molecule that is larger than the mature enzyme and the nascent proenzyme undergoes N-linked glycosylation, most likely cotranslationally. However, prior to the action of Golgi mannosidases on the oligosaccharide processing of pro-MPO is completed without formation of complex-mannose side chains. The pro-MPO enters one or more prelysosomal compartments in which it is proteolytically cleaved into mature MPO. Additional studies are needed to determine the role of the mannose-6-phosphate receptor and alternative pathways for the intracellular transport of myeloid lysosomal enzymes such as MPO.

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HL-T, A New Cell Line Derived From HL-60 Promyelocytic Leukemia Cell Cultures Expressing Terminal Transferase and Secreting Suppressor Activity

By Elisabeth Paietta, Richard J. Stockert, Theresa Calvelli, Peter Papenhausen, Stephanie V. Seremetis, Marilena Fotino, Peter H. Wiernik, Lucy Chang, and Fred J. Bollum

A cell line with immature blast cell morphology was isolated from HL-60 promyelocytic leukemia cell cultures and designated HL-T. This new cell type is biphenotypic, expressing terminal transferase (TdT) together with myelomonocytoid immunologic features. TdT enzymatic activity, undetectable in HL-60, was determined to be 140 to 180 units/10^8 HL-T cells by the dGTP-assay, approximately 20% of the activity found in lymphoblastoid cell lines. HL-T predominantly synthesize the known 58-kDa TdT-protein plus a minor 54/56-kDa doublet. The 58-kDa steady state form is nonglycosylated and is phosphorylated. Precursor antigens S3.13 and MY-10, absent on HL-60, are expressed by HL-T; however, the cells are negative for HLA-DR. Southern blot analysis by hybridization with immunoglobulin heavy chain (JH) and T cell-receptor chain gene (Tβ) probes shows JH to be in the germ-line configuration in both cell lines and the Tβ gene to be in germ-line in HL-60 but to be rearranged in HL-T. Truncation of the gene encoding the granulocyte–macrophage–colony-stimulating factor (GM-CSF), as found in HL-60, is not observed in HL-T. HL-T are resistant to differentiation–induction by retinoic acid and 1,25-dihydroxyvitamin D_3. Cytogenetically HL-T share with HL-60 a deletion of the short arm of chromosome 9 at breakpoint p13, an aberration frequently found in patients with T cell leukemia. In addition, HL-T display t(8;9)(p11;p24) and trisomy 20. Tetraploidy is observed in 80% of HL-T metaphases with aberrations identical to those in the diploid karyotype. Like HL-60, the new line shows some surface-antigenic–T cell characteristics. Despite an antigenic pattern most consistent with that of helper-inducer T cells ($T4^+$, $D44^\pm$, $4B4^+$, $2H4^-$, $TQ1^\pm$), HL-T cells and their conditioned culture medium suppress antigen, mitogen, and mixed-leukocyte–culture-mediated lymphocyte proliferation.

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THE HL-60 CELL LINE has been established from the peripheral blood of a patient with acute promyelocytic leukemia.[1] Morphologically, histochemically, and immunologically, the cell population consists predominantly of promyelocytes with a small number of more mature myeloid elements present, reflecting a low rate of spontaneous maturation. These cells have attracted wide interest because of their potential to respond to various agents by differentiation in vitro along the myeloid (eg, induced by retinoic acid),[2] the monocytic (eg, induced by 1,25-dihydroxyvitamin D_3),[3] or eosinophilic lineage (eg, in response to butyric acid and alkaline conditions).[4] In one instance, commitment of HL-60 cells toward nonlymphoid cells was suspected from the reactivity with null lymphoblasts of antibodies raised against phorbol ester-induced HL-60 cells.[5] Inhomogeneity of the HL-60 cell population is suggested by the selection of differentiation-resistant subclones through growth in the presence of inducer drugs.[6]

We have observed the spontaneous outgrowth from HL-60 cultures of a cell line with immature blast cell morphology, HL-T. A parental relationship of this new cell type with HL-60 cells is discussed in view of antigenic, cytogenetic, and functional similarities between the lines. The unique features of HL-T as a TdT^+/myelomonocytic biphenotype with T cell-receptor β chain gene rearrangement and the antigenic profile of $T4^+$ helper-inducer cells despite marked suppressor activity should make this line a valuable tool for various aspects in hematopoietic research.

MATERIALS AND METHODS

Cell cultures HL-60 cells, as the counterpart for the variant HL-T cells, were provided by Dr R. Gallagher at Montefiore Medical Center, Bronx, NY. The HL-60 culture from which the HL-T line arose was purchased from the American Type Culture Collection, Rockville, MD. Cell lines were maintained in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 20% fetal bovine serum, 0.3% L-glutamine, 100 μg/mL streptomycin, and 100 U/mL penicillin in a humidified atmosphere of 5% CO_2 in air at 5×10^5 and 2×10^6 cells/mL. For experimentation harvested cells were freed of debris and dead cells by Ficoll-Hypaque (FH) density centrifugation.

In vitro induction of differentiation Late log-phase HL-T or HL-60 cells were washed twice to remove spent medium and cultured at 5×10^5 cells/mL of complete culture medium containing 10^{-6} mol/L retinoic acid (Sigma, St. Louis) or 10^{-7} mol/L 1,25-dihydroxyvitamin D_3 (generously provided by Hoffmann-La Roche, Nutley, NJ) for four days before phenotypic changes were evaluated. Control cultures contained the appropriate ethanol concentrations.

Electron microscopy studies Cell pellets were fixed in 2.5% glutaraldehyde in 0.067 mol/L phosphate buffer, pH 7.4, postfixed

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in Dalton's chromic osmium, dehydrated in ethanol, and embedded in Epon after two changes in propylene oxide. Thin sections were stained with uranyl and lead salts and viewed in a Siemens 101 electron microscope.

Cytogenetics. Cytogenetic analysis was done on unstimulated cells using the methotrexate synchronization technique[7] and trypsin-Giemsa banding. Twenty metaphases were examined for each cell line.

Immunophenotyping. A series of mouse monoclonal antibodies (MoAbs; Table 1) was tested by indirect immunofluorescence with fluoresceinated affinity-purified F(ab')$_2$ fragment–goat antimouse immunoglobulins (Cappel Laboratories, Cooper Biomedical, Inc,

Table 1. Immunophenotype

Antibodies	Source	Cluster Designation (CD) Specificities	Percent Fluorescent Cells* HL-60	Percent Fluorescent Cells* HL-T
Polyclonal rabbit antisera				
TdT	(a)	Immature lymphocytes[8,9]	0	60-70
Monoclonal murine antibodies				
Stem cell markers				
S3.13	(b)	CFU-GEMM-myeloblast, mono, T cell[10]	13	95
MY-10	(c)	CD34, CFU-GM, monoblast[11]	28	95
S8.6	(b)	CFU-GEMM-promyelocyte, mono[10]	5	0
S16.144	(b)	CFU-GEMM-metamyelocyte, mono[10]	95	95
MY-9	(d)	CD33, CFU-GEMM-metamyelocyte, mono[11]	89	0
RFDR-1	(e)	HLA-DR[12]	0	0
VIP-2b	(f)	CD38,T10, committed stem cells[13,14]	0	37
Myelocytic monocytic markers				
S4.7	(b)	CFU-GM-neutrophil, mono[10]	74	90
VIM-2	(f)	Myeloblast-neutrophil, mono[11]	95	17
VIM-D5	(f)	CD15, promyelocyte to neutrophil[11]	95	5
VIM-12 – OKM1	(f)	Myelocyte to neutrophil, mono[14]	10	0
VIM – 13	(f)	CD14, MO2-antigen, mono[15]	6	0
903 – Mo1	(d)	CD11, MO1-antigen, PMN, mono[14]	0	0
RFD-9	(e)	Macrophages, B, and erythroid cells[16]	80	95
T lymphoid markers				
OKT6	(g)	CD1, thymocyte antigen[11]	82	0
OKT11	(g)	CD2, pan-T[11]	0	1
OKT3s*	(g)	CD3, mature T cells[11]	75	0
OKT3c*	(g)	TdT$^+$ thymic blasts[17]	0	0
Leu-1	(h)	CD5, pan-T[11]	0	90
RFT-12	(e)	CD6, peripheral T cells[12]	0	30
RFT-2	(e)	CD7, pan-T[12]	5	87
Leu-3a	(c)	CD4, helper T cells[11]	52	94
OKT4a	(g)	CD4, helper T cells[11]	83	83
Anti-T4	(i)	CD4, helper T cells[11]	86	90
D44	(k)	Corticothymocyte, helper, NK cells[18]	43	30
4B4†	(i)	CDw29, helper–inducer cells[19]	96	98
Leu-2a	(h)	CD8, suppressor cells[11]	2	1
2H4†	(i)	Suppressor–inducer cells[19]	83	0
TQ1†	(i)	Suppressor–inducer cells[19]	74	25
VIP-1b	(f)	T9 antigen, transferrin-receptor[13]	92	71
B lymphoid markers				
VIL-A1	(f)	CD10, common ALL antigen[20]	0	0
RFB-7	(e)	CD20, B1 antigen, pan-B[12]	2	0
VIB-C5	(f)	CD24, BA-1 antigen[14]	5	0
NK markers				
Leu-7	(h)	T cell and NK subset[11]	0	0
VEP-13	(f)	CD16, Fc IgG receptor on NK, neutrophils[11]	78	0

Both cell lines were negative for erythroid and megakaryocytic antigens.

The letters in brackets refer to the sources by whom the reagents are made or distributed: (a) F.J. Bollum, Bethesda, MD, (b) G. Rovera, The Wistar Institute, Philadelphia; (c) R. Tindle, Sera-Lab, Sussex, UK; (d) J.D. Griffin, Dana Farber Cancer Institute, Boston; (e) G. Janossy, Royal Free Hospital, London; (f) W. Knapp, Institute for Immunology, University of Vienna, Austria; (g) Ortho Diagnostics Systems, Raritan, NJ, (h) Beckton-Dickinson, Mountain View, CA; (i) Coulter Immunology, Hialeah, FL; (k) A. Bernard, Institut Gustave-Roussy, Villejuif, France.

Abbreviation: mono, monocytes.

*Fluorescent cells were analyzed with a FACStar flow-cytometry system (except for TdT-staining)

†These antibodies recognize widely distributed structures so that their designation as functional antigens needs to be considered cautiously.

Malvern, PA) as second antibodies. Intracytoplasmic T3 antigen was detected on methanol-fixed (30 minutes at 4°C) cytospin preparations using the VIT-3 antibody (40 minutes at 22°C). Background fluorescence was determined with P3X63NS1 myeloma culture supernatant with added mouse IgG at 100 μg/mL (Cappel). Fluorescent cells were assessed either manually, using a Nikon microscope (Nikon Inc, Garden City, NY) with incident illumination and equipped for the dual wavelength method, or by flow cytometry (Beckton-Dickinson FACSTAR, Mountain View, CA), using an argone laser emitting 170 mW at 488 nm. Control samples consistently gave 5% or less of positive cells. Narrow-angle forward light scatter was used as a measure of cell size and for excluding debris. Fluorescence intensity was measured on log scale.

HLA-typing of the cell lines was done by a microcytoxicity method using complement that had been extensively absorbed with either cell line.[21]

Terminal transferase (TdT) TdT activity was detected microscopically in methanol-fixed cells (30 minutes, 4°C) by indirect immunofluorescence using monospecific rabbit antiserum and fluorescein-conjugated $F(ab')_2$ goat antirabbit IgG as second antibody.[22] For two-color immunofluorescence, myeloid-specific antibodies were counterstained with phycoerythrin-conjugated streptavidin via biotinylated affinity-purified $F(ab')_2$ fragment–goat antimouse Ig (Beckton-Dickinson, Mountain View, CA) and combined with TdT staining. For immunoblotting, polyclonal anti-TdT antiserum was used at 15 μg IgG/mL of incubation mixture; preimmune rabbit serum served as control. A cocktail of four monoclonal anti-TdT antibodies, HTdT-1, -2, -3, and -4,[23] (10 μL equal to 0.5 μg of total IgG) or control Cappel supernatant media was employed in immunoprecipitation. The combination of all four monoclonal HTdT antibodies is required to fully reproduce the fluorescence intensity obtained with the rabbit heterologous anti-TdT antiserum.

TdT enzymatic activity was determined as described.[24] Cells (4×10^7), washed with RPMI 1640 medium, were lysed in 160 μL of 0.313 mol/L phosphate buffer, pH 7.4, by freeze-thawing. The lysate was cleared at 175,000 g for 45 minutes and aliquots assayed using 5 μmol/L $d(pA)_{50}$ as initiator, 1 mmol/L ^{3}H-dGTP (44 cpm/pmol) as substrate, 8 mmol/L $MgCl_2$, 0.2 mol/L potassium cacodylate buffer at pH 7.2 and 1 mmol/L 2-mercaptoethanol. Products of the reaction were scored as acid-insoluble radioactivity on glass fiber filters. One unit of enzyme activity is defined as 1 nmol dGMP incorporated in 60 minutes at 36°C.

DNA analysis. DNA was extracted from lysed HL-60 and HL-T by proteinase K digestion, phenol extraction, and ethanol precipitation according to established procedures.[25] For hybridization with cloned TdT complementary DNA (cDNA), cellular DNA (15 μg) was digested with the restriction enzymes Pst I, HindIII, or BamHI (Bethesda Research Laboratories, Gaithersburg, MD), sized in 1% agarose gels, transferred to nitrocellulose sheets, and hybridized according to Southern[26] with the nick-translated probes pT711 and pT106, spanning the total coding sequence of TdT.[27] For studying immunoglobulin and T cell receptor–β chain (Tβ) gene rearrangements, DNA was digested with EcoRI or BamHI restriction endonucleases, electrophoresed in 0.8% agarose gels, transferred, hybridized, and processed as described.[28] The Tβ probe used was derived from a human cDNA Tβ clone (YTJ-2) isolated from the Jurkat-2 T lymphoma cell line (a gift from Dr T. Mak, University of Ontario Cancer Institute, University of Toronto, Canada). This clone hybridizes to both alleles (Cβ1 and Cβ2) of the constant (TβC) region and to one or more alleles of the variable (V) regions.[28] The probe for the immunoglobulin locus, representative of the J region (JH) of the heavy chain (IgH) locus, was obtained from Dr Stanley Korsmeyer (National Institutes of Health, Bethesda, MD).[29] Studies on the genomic organization of the GM-CSF gene were performed by Dr James Ihle, Frederick Cancer Center, Frederick, MD, using the probe pCH5.2, a 5.2-kb HindIII fragment of genomic DNA containing three exons of the GM-CSF gene cloned in pBR322.[30]

Immunoblotting. Cell aliquots (10^6 cells/200 μL) and prestained mol wt (Bethesda Research Laboratories) were heated at 100°C for ten minutes in SDS-sample buffer containing 1 mmol/L dithiothreitol, proteins resolved on 10% SDS-polyacrylamide gel electrophoresis (PAGE),[31] electrophoretically transferred to nitrocellulose paper,[32] and processed as described[33] using 100μL of polyclonal anti-TdT antiserum for probing and iodinated protein A to detect protein-bound immunoglobuin on the nitrocellulose paper.[34] The nitrocellulose sheet was blotted with paper towels, air dried, and exposed to Kodak XAR-5 x-ray film for 18 hours at −70°C.

L-^{35}S methionine metabolic labeling. Cells were methionine-deprived for 30 minutes at 37°C before 500 μCi of L-^{35}S methionine (specific activity 1,110 Ci/mmol; Amersham, Arlington Heights, IL) were added for three hours at 37°C. Cells were then washed with serum-containing RPMI 1640 medium and lysed in ice-cold lysis buffer (0.1 mmol/L Naphosphate, pH 7.2, 1% NP40, 2.5 mmol/L phenylmethylsulfonyl, 1mmol/L EDTA). The cleared supernatant was prepurified with 10 μg of mouse IgG and 50 μL of a 1:20 suspension of protein A Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ) before 10 μL of monoclonal anti-TdT antibody cocktail was added overnight at 4°C. Antigen–antibody complexes were recovered with 50 μL of protein A sepharose CL-4B, released into reducing sample buffer as described and resolved on 10% SDS-PAGE.[34] Gels were fixed, impregnated in Enhance (New England Nuclear, Boston, MA) for 60 minutes, dried, and fluorographed at −70°C for 1 week using Kodak SB 5 film.

To test for glycosylation of the TdT protein, cells were grown in the presence of 2 μg/mL tunicamycin (Boehringer Mannheim, Indianapolis, IN) for four hours. This antibiotic is classically used to inhibit cotranslational protein N-glycosylation.[35]

Phosphorylation of the TdT protein. Cells were preincubated in phosphate-free Eagle's minimum essential medium (GIBCO) for 15 minutes at 37°C before $[^{32}P]P_i$ (carrier-free; Amersham) was added at 1 mCi/mL.[36] Cells were washed with ice-cold phosphate-buffered saline (PBS), solubilized, and subjected to immunoprecipitation. Immunoprecipitates were resolved on 10% SDS-PAGE followed by fluorography as described above.

Functional studies The proliferative response to mitogens in vitro of normal peripheral blood lymphocytes isolated by Ficoll-Hypaque density centrifugation was measured by incorporating ^{3}H-thymidine during six hours after stimulation for 72 hours with the mitogens phytohemagglutinin (0.1%), concanavalin A (con A, 10 μg/mL), pokeweed mitogen (PWM, 0.01%), and phytohemagglutinin plus interleukin 2 (10%; Cetus, Emeryville, CA) or for 120 hours with the specific antigens Candida (2 units), PPD (2.5 μg) or tetanus toxoid (340 μg) with the automatic cell harvester Mash 2. For mixed lymphocyte cultures[37] HL-T and HL-60 cells were treated with mitomycin C (30 μg/mL/10^6 cells, 20 minutes, 37°C), washed in PBS, and added to lymphocyte cultures at 7.5×10^4 cells per well (200 μL), if not indicated otherwise. Conditioned media from three-day HL-60 or HL-T cultures (inoculated at 10^7 cells/10 mL) were filtered (0.45 μm) and used at a ratio of 1:1 with 10% fetal bovine serum (FBS) containing RPMI 1640 medium. Viability of lymphocytes in the presence of 50% HL-T-conditioned medium was assured by measuring the spontaneous release of radioactivity from ^{51}Cr-labeled lymphocytes cultured for 18, 36, and 72 hours as compared with culture in control medium.

Natural killer (NK) and lymphokine-activated killer (LAK) cell activity were studied in a four-hour chromium release assay using the erythroleukemia cell line K562 as natural killer-sensitive target and the lymphoblastoid cell line Daudi (both provided by Dr K.

Welte, Memorial Sloan-Kettering Cancer Center, New York) and fresh tumor cells isolated from the ascites fluid of patients with solid tumors as LAK-cell targets. Target cells (2×10^6 cells/0.5 mL) were labeled with 200 μCi $Na^{51}CrO_4$ (specific activity 490 to 670 mCi/mg, New England Nuclear) at 37°C for one hour, washed, incubated for an additional 30 minutes, filtered through 110-mesh Nytex filter, and distributed in round-bottom 96-well dishes at 10^3 cells/well (50 μL). To these, the effector cells (HL-60, HL-T) were added (100 μL) at effector·target cell ratios 40.1, 10:1, and 2.5:1. Spontaneous release was measured in wells containing only target cells and medium. Total release was obtained by adding 2% SDS. After four hours of incubation at 37°C in 5% CO_2 in air, the well supernatants were harvested in a Skatron-Titertek System (Skatron AS, Sterling, VA) and counted in a gamma counter. Lytic activity is expressed as the mean percentage of ^{51}Cr released in triplicate assays.

To test for induction of LAK-cell activity, HL-T cells were cultured with 1,500 units of recombinant interleukin 2 (Cetus) for four days in RPMI 1640 medium with 20% FBS. Expression of the interleukin 2 receptor was evaluated using the anti-Tac MoAb (provided by Dr T.A. Waldmann, NCI, Bethesda, MD).

RESULTS

Phenotype. HL-T cells express the morphology of immature myeloblasts with a high nuclear-cytoplasmic ratio, loose chromatin texture, occasionally inconspicuous nucleoli, and basophilic, vacuolated cytoplasm without granulation. Electron microscopy, performed to reveal microgranules, as seen in some cases of acute promyelocytic leukemia,[38] detected neither granulation nor viral particles. HL-T are negative for special histochemical stains including myeloperoxidase, α naphthyl butyrate, chloroacetate esterase, and periodic acid-Schiff reaction.

Testing for HLA antigens revealed HLA-A1, A2, B22, B17, Bw4, Bw6, Cw6 plus weak reaction with typing antisera defining B8 and B40 for HL-60, and HLA-A1, A19/31, B8, B40, Bw6 for HL-T, indicating that, with respect to their predominant HLA markers, the lines are only remotely related.[39]

Karyotype. Cytogenetic analysis of HL-T revealed tetraploidy in 80% of the metaphases, 94,XXXX,9p-(p13),t(8;9)(p11;p24),+20,+20, and a diploid karyotype with identical abnormalities in 20% of the cells (Fig 1). Compared with initial passages 10 months ago, the percentage of tetraploid cells has since then increased from 50% to 80%. HL-T share with HL-60 a rare deletion of the short arm of chromosome 9 at breakpoint p13. Cytogenetic analyses of HL-60 at earlier and later passages performed in our laboratory and reported by others[1,40-42] show chromosomal instability. In the most recent analysis we find two clones, 45,X0,9p-(p13),t(10;13)(p11;q11),17p+,−5,+18 in 45% of

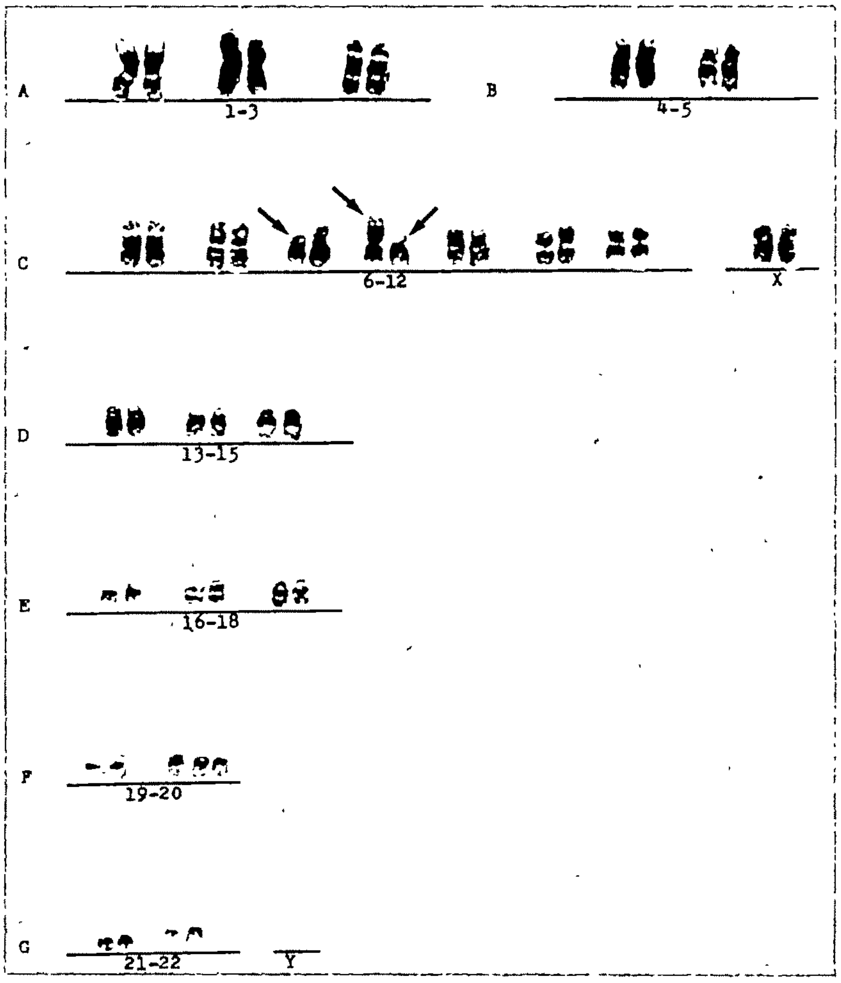

Fig 1. Banded karyotype of a diploid metaphase of HL-T cells. The two characteristic abnormalities, del 9 (p13) and t(8;9) are indicated by arrows.

the metaphases and in the 10% of hypotetraploid cells; an additional 4q+ is present in the rest of the hypodiploid cells.

As expected from the differential karyotypes of HL-60 and HL-T, both alleles of the gene encoding GM-CSF are unrearranged in HL-T cells, which have two intact chromosomes no. 5, while the well-described[30] partial deletion of the GM-CSF gene was found in HL-60 cells.

Terminal transferase (TdT). By indirect immunofluorescence, 60% to 70% of HL-T cells express the lymphoid-specific enzyme TdT. Enzymatic activity in HL-T is 140 to 180 units/10^8 cells using dGTP as substrate or 20 to 25 units/10^8 cells using dATP, consistent with a higher sensitivity of the dGTP assay.[24] In comparison, the lymphoblastoid cell line RPMI 8402 (100% of TdT$^+$ cells by immunofluorescence) contains 160 to 200 dATP units/10^8 cells. This suggests a lower level of enzymatic TdT activity per HL-T cell compared with lymphoblastoid cells. HL-60 cells are negative for TdT by both assays.

In metabolically labeled HL-T, anti-TdT antibodies recognize a major 58-kDa protein plus a minor 54/56-kDa doublet. The 58-kDa protein corresponds to the high mol wt form of TdT found in various lymphoid tissues.[23,43,44] Lower mol wt peptides of TdT of 32 kDa and below have been attributed to proteolytic degradation.[45] As seen in Fig 2, such TdT-specific bands did not appear during the three-hour synthesis period in HL-T cells investigated. Whether the 54/56 kDa pair represents beginning degradation or a new product remains unclear at present. By immunoblotting, the 58-kDa protein was confirmed as the steady state form of the enzyme; it is nonglycosylated and possesses a phosphorylation site (data not shown), both characteristic features of the enzyme[27,44] (F.J. Bollum, unpublished findings). We were unable to demonstrate the presence of TdT proteins in HL-60 cells either by metabolic labeling or by immunoblotting.

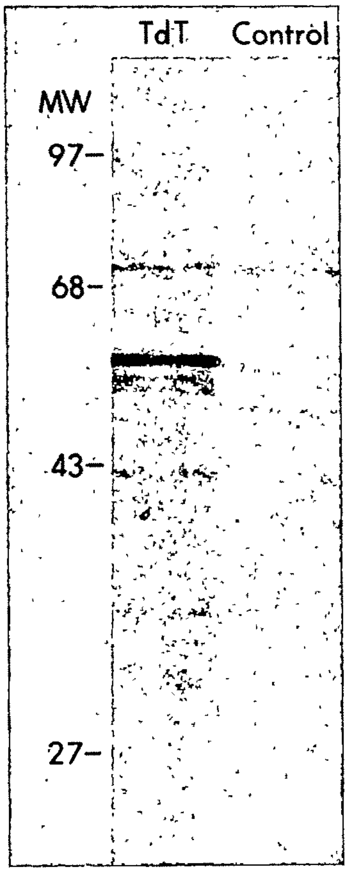

Fig 2. Fluorograph of 10% SDS-PAGE of TdT proteins in HL-T. Immunoprecipitation from extracts of ^{35}S-methionine labeled HL-T cells with a cocktail of monoclonal anti-TdT antibodies (Lane TdT) or with control mouse immunoglobulin demonstrates a TdT-specific 58-kDa band plus a 54/56-kDa doublet. All additional proteins were precipitated nonspecifically. MW, mol wt standards.

Blot hydridization of HL-T and HL-60 DNA with the cloned TdT–cDNA inserts pT71·1 and pT106 revealed no difference in the restriction enzyme-fragment patterns for genomic TdT sequences in the two cell types (Fig 3).

Immunophenotype. The reactivity patterns of HL-T and HL-60 cells with a series of MoAbs are compared in Table 1. HL-T coexpress TdT and myelomonocytic antigens recognized by S16.144 and S4.7, neither of which has ever been demonstrated on lymphoid cells[10] but are unreactive with MY-9, an antibody with overlapping specificity.[10,11] HL-T express the precursor cell antigens S3.13 and MY-10, both lacked by HL-60. The presence of S3.13 antigen on HL-T may reflect either immaturity, monocytic nature, or close

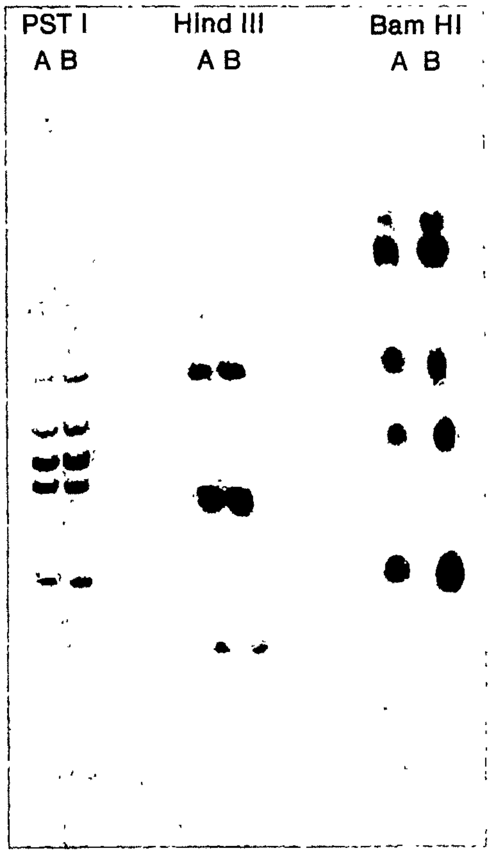

Fig 3. Hybridization of the human TdT cDNA probes pT711 and pT106 to DNA from HL-60 (lane A) and HL-T cells (lane B) cleaved with restriction enzymes PstI, Hind III, or Bam HI.

affiliation with the T cell lineage.[10] In favor of the latter, HL-T lack another very early hematopoietic antigen not expressed by T lymphocytes,[10] the S8.6 antigen. The distribution of MY-10 on normal and leukemic cells parallels the expression of HLA-DR, except that monocytes and B cells are MY-10 negative.[11] Hence the finding of MY-10 on the HLA-DR negative HL-T is without precedent. HL-T virtually lack antigens of myeloid cells at or beyond the stage of myeloblasts, whereas HL-60 express myeloid-associated antigens, including the X-hapten first appearing at the promyelocyte stage. The M2 antigen, associated with the myeloblast stage,[11] is only detected on 17% of HL-T cells, which in double staining are TdT$^+$. Most interestingly, both HL-T and HL-60 express the D9 antigen specific for activated macrophages and erythroblasts[16] but lack other monocytic or erythroid antigens. In our experience this antigen is present on blast cells from patients with acute monocytic leukemias but is absent from leukemic promyelocytes (Paietta et al, unpublished). HL-T and HL-60 both express T cell characteristics. Although the lines are negative for CD2 (T11), the sheep RBC receptor, they express the T4 antigen as detected by three different MoAbs. While HL-60 cells additionally express the thymocyte antigen CD1 (T6) and the mature T cell marker CD3 (T3), HL-T react with two pan-T antibodies, anti-CD5 and anti-CD7, but are negative for CD1 and CD3. With respect to functionally related antigens, HL-T are negative for NK-specific antigens and express predominantly the phenotype of helper–inducer cells (4B4$^+$, D44$^\pm$, 2H4$^-$, TQ1$^\pm$). HL-60 cells express the NK cell antigen CD16 and both helper–inducer (4B4$^+$, D44$^\pm$) as well as suppressor–inducer antigens (2H4$^+$, TQ1$^+$).

JH and Tβ gene rearrangement. For analysis of the genomic organization of the Tβ and IgH in HL-60 and HL-T, EcoRI or BamHI DNA digests[28] were tested in Southern blot hybridization using Tβ and JH probes. As a normal control, human fibroblast DNA was used. Whereas HL-60 cells have germ-line configurations of both Tβ (Fig 4) and IgH genes (not shown), HL-T display a rearrangement of the Tβ gene, while the IgH gene is unrearranged.

Functional activity. Mitomycin C-treated HL-T cells strongly suppress mitogen-induced lymphocyte proliferation, and conditioned HL-T culture medium is an even more potent suppressor (Fig 5 A). ^{51}Cr-release studies with labeled lymphocytes over 72 hours in the presence of 50% HL-T-conditioned medium confirmed that this suppression was not due to a lymphotoxic effect of the medium. Neither HL-60 cells nor culture supernatant demonstrate a comparable effect. When tested in antigen-induced lymphocyte proliferation, both HL-T and HL-60 induce suppression, although the HL-T effect is more pronounced (Fig 5 B). Furthermore only HL-T cells inhibit the mitotic response obtained in three-way mixed lymphocyte culture. Supernatant media of both HL-T and HL-60 cultures strongly suppress this proliferation (Table 2). Neither cell line exhibits NK or LAK cell activity. Culturing HL-T with interleukin 2 for four days did not induce expression of the interleukin 2 receptor or generate LAK cell activity.

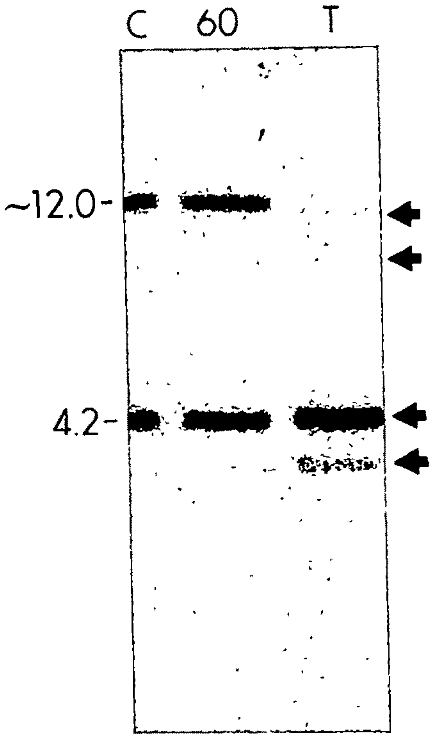

Fig 4. Southern blot analysis of HL-60 (lane 60) and HL-T (lane T) DNA with Tβ probe. Genomic DNA was digested with EcoRI and probed with Tβ. Lane C represents an identical Southern analysis of germ-line DNA obtained from human fibroblasts. The sizes of the restriction fragments detected by the Tβ probe in the control, that is, the germ-line bands at 11 to 12 and 4.2 kb pairs, are denoted by the solid lines on the left side of the figure. The arrows on the right side mark the location of rearranged bands in HL-T.

As a putative derivative of the inducible HL-60 cells, we tested HL-T for responsiveness to retinoic acid and 1,25-dihydroxyvitamin D$_3$, two effective inducers of HL-60 differentiation.[2,3] Exposure of the cells to either agent did not alter morphology, TdT activity, or expression of surface antigens.

DISCUSSION

We describe the isolation of a new cell type from cultures of the human promyelocytic leukemia cell line, HL-60. Emergence of the line followed a period of growth cessation and spontaneous maturation of the HL-60 culture. Although this new line, designated HL-T, shares with HL-60 some antigenic, cytogenetic, and functional features, its origin remains to be established. With respect to the HLA-phenotype, the two lines differ at the A and B loci. However, the weak reactivity of HL-60 cells with antisera defining the major B antigens of HL-T may reflect the presence of a subclone of cells within the parental cell line that expresses an HLA type similar to that of HL-T. It is remarkable that another HL-60 subclone, BII,[46,47] although distinct from HL-T with respect to karyotype and immunophenotype, is closely related to HL-T with respect to the HLA type, with both lines expressing HLA-A1, A31, and B40. While such trivial explanations as contamination or chimerism[48] cannot be excluded, the observed genetic instability of the HL-60

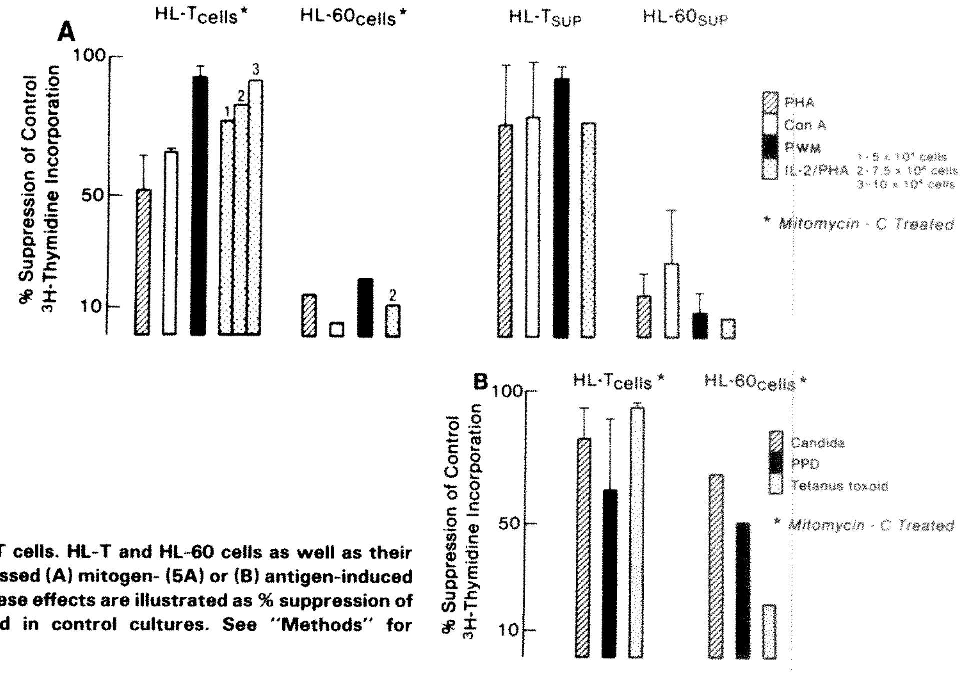

Fig 5. T cell function of HL-T cells. HL-T and HL-60 cells as well as their conditioned culture media suppressed (A) mitogen- (5A) or (B) antigen-induced lymphocyte proliferation (5B). These effects are illustrated as % suppression of the mitotic responses measured in control cultures. See "Methods" for details.

parental line is the most plausible explanation for the presence of both the BII and the HL-T subline.

By cytogenetic analysis, both HL-60 and HL-T possess a rare deletion on the short arm of chromosome 9 at break point p13, an abnormality frequently present in acute lymphoblastic leukemia (ALL) with T cell features and poor prognosis.[49-51] Such blast cells often lack the receptor for sheep RBCs despite the expression of other T-associated antigens,[50,51] as is true for HL-T, but lack HLA-DR and TdT, consistent with a more mature T cell phenotype.[50,51] However, 9p- together with trisomy 20, another cytogenetic aberration in HL-T, has been detected in the T lymphoblastoid, TdT$^+$ cell line CCRF-CEM.[52] Although 9p- is generally affiliated with a lymphoid phenotype, one case with biphenotypic features has been reported.[50] Chilcote et al[53] found 9p- to be associated with an inactivation of the enzyme methylthioadenosine phosphorylase, resulting in an increased sensitivity of such blast cells to agents that inhibit de novo purine synthesis.

Like others,[1,40-42] we have found marked chromosomal instability of HL-60. Most consistently, the cells are predominantly hypodiploid (modal chromosome number 45) with −X, del 9 (p13), −5, +18, a rearrangement between chromosome 17 and some unidentified material, and t(10;13)(p11;q11). Since the gene for human TdT is localized on chromosome 10,[54] the translocation involving chromosome 10 present in HL-60 but absent in HL-T prompted us to compare their genomic organization for TdT. Although no gross differences were detected, point mutations cannot be excluded.

Expression of TdT in acute nonlymphoid leukemias is frequently associated with clonal immunoglobulin or T cell receptor–gene rearrangements.[28,55] Our finding in the lineage of promiscuous, TdT$^+$, HL-T cells of a rearranged Tβ gene further supports the suggested putative role of TdT in lymphocyte ontogenesis.[56] Since during intrathymic differentiation T cell receptor genes are rearranged and expressed synchronous with certain surface antigens,[57] the expression by HL-T of CD5 but not of CD2 or CD3 localizes an arrest of the T lymphoid differentiation program in these cells after the Tβ gene rearrangement.

Inhomogeneity of the HL-60 cell population is further suggested by the possible selection of sublines for their ability to continue growing in the presence of either differentiation-inducing agents[58] or of cytostatic drugs.[59] Resistance has been in part related to alterations in cellular drug metabolism[58,59] or protein glycosylation[60] and in part to the gain or loss of chromosomes.[58,61] The high level of tetraploidy in

Table 2. Effect of HL-T and HL-60 on Three-Way Mixed Lymphocyte Culture Proliferation

Proliferative Responses (100%)	$HL\text{-}T_{cellsM}$ % Decrease	$HL\text{-}60_{cellsM}$ % Decrease	$HL\text{-}T_{sup}$ % Decrease	$HL\text{-}60_{sup}$ % Decrease
Donor A_M + donor B	37.2 ± 8*	0†	78.0	64.8
Donor A + donor B_M	46.0 ± 17*	19.6	90.0	56.8
Donor A + donor B	55	0‡	87.2	72.3

Values are derived from single experiments or are given as the mean ±SEM from two experiments.

Abbreviations: M, mitomycin C-treated cells.

*Not-mitomycin C-treated HL-T cells suppressed the proliferative responses by 81% and 86.2% in the case of donor B and donor A, respectively.

†,‡Mitomycin-treated HL-60 cells increased the proliferative response by 52% and 28.9%, respectively.

HL-T cells may be related to their lack of responsiveness to differentiation induction by retinoic acid and 1,25-dihydroxyvitamin D_3.

As apparent from Table 1, the immunophenotype of HL-T is unique. The cells express TdT, reflecting immature lymphoid features, but lack HLA-DR, CD1, or common acute lymphoblastic leukemia antigen (CALLA). Positivity for D44, an antigen present at high density on cortical thymocytes (and peripheral T cells), for S3.13 and MY-10 antigen, although indicative of a precursor differentiation state, is aberrant in view of the other antigenic characteristics. That the recognition of TdT in HL-T cells by indirect immunofluorescence is not attributable to nonspecific interaction of the heteroantiserum with proteins other than TdT, as found in phytohemagglutin-stimulated lymphocytes,[62] is confirmed by measurable enzymatic activity, immunoprecipitation from metabolically labeled cells, and immunoblotting.

HL-T cells coexpress lymphoid and myeloid antigens and are therefore to be considered biphenotypic. Only two other such cell lines have been described to date: RS4;11 with B lineage and monocytic features and the t(4;11) typically associated with this cell type in a subgroup of pediatric acute lymphoblastic leukemia[63] and RED-3, positive for TdT, T4 antigen, the myeloid-associated X-hapten and myeloperoxidase, which upon induction with retinoic acid differentiate along the myeloid cell lineage.[64] RED-3 and HL-T clearly differ with respect to the expression of the X-hapten, an antigen not expressed before the stage of promyelocytes during normal hematopoiesis.[34] The finding of T antigens, especially T4, on otherwise myeloid cells is not uncommon.[65] In the HL-60 cells, however, the antigenic profile appears to be more complex in that both the thymocytic antigen CD2 and the mature T antigen CD3 are present (Table 1). We have described a patient with TdT^+ acute promyelocytic leukemia with blast cells positive for CD1 and developing CD3 upon differentiation induction in vitro.[66] Since then we have observed a minor TdT^+ component in six of eight patients with acute promyelocytic leukemia.[67] These data support a closely related differentiation potential of promyeloblasts and T lymphoblasts.

The TdT positivity of HL-T, suggesting an immature lymphoid differentiation state, should preclude cell-mediated lymphocyte function.[68] Instead, strong suppression of both T-dependent (mitogen-induced)[69] and T-independent lymphocyte proliferation (antigen-stimulated, three-way mixed leukocyte reaction)[69] was observed with HL-T; with HL-60 cells, only the latter activity was seen (Figs 5A and 5B). These suppressor activities were not predicted by the antigenic phenotype, which is most consistent with that of helper–inducer cells in the case of HL-T and with that of suppressor–inducer cells in the case of HL-60, even though the antibodies 4B4, 2H4, and TQ1 recognize broadly distributed structures. The simultaneous expression of CD16 and CD3 antigens by HL-60 cells is consistent with their being effector cells in antibody-dependent cellular cytotoxicity[70] without significant NK cell activity. This antigenic and functional profile is characteristic of a unique, small, subset of peripheral blood T lymphocytes.[71]

The potent suppressive activity present in the conditioned medium of HL-T cells, and to a lesser extent in HL-60 culture medium, suggests the production and secretion of an inhibitory factor reminiscent of soluble suppressor lymphokines recently described in T cell hybridoma supernatants.[72] Whether the suppression of lymphocyte proliferation resides in a lymphokine unique to HL-T cells has to await its isolation and characterization.

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Adenosine Dialdehyde and Nitrous Oxide Induce HL-60 Differentiation

By Renate B. Pilz, Georges Van den Berghe, and Gerry R. Boss

Adenosine dialdehyde and nitrous oxide, specific S-adenosylhomocysteine hydrolase and methionine synthetase inhibitors, respectively, induced differentiation of the human promyelocytic cell line HL-60. Their effect did not appear to be mediated through changes in transmethylation or decreased S-adenosylmethionine synthesis because (1) there was little correlation between the concentrations of adenosine dialdehyde that induced differentiation and those that changed the ratio of the intracellular concentrations of S-adenosylmethionine to S-adenosylhomocysteine, and (2) inhibition of methionine adenosyltransferase by cycloleucine did not induce differentiation. The differentiation induced by adenosine dialdehyde was prevented by homocysteine and that by nitrous oxide was inversely related to the medium methionine concentration. This suggested that differentiation was secondary to decreased methionine synthesis.

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A BETTER UNDERSTANDING of the regulatory mechanisms governing cellular differentiation could lead to new forms of therapy of acute leukemias.[1] The human promyelocytic leukemia cell line HL-60 is a valuable tool for studying differentiation in vitro because it can be induced to differentiate into morphologically and functionally mature metamyelocytes and granulocytes.[2] There are numerous unrelated compounds that induce HL-60 differentiation.[2-5] Although each agent must in some way change gene expression, their mechanisms of action are poorly understood and this relates, in part, to their multiple metabolic effects.[6] We decided to approach this problem by using agents with well-defined sites of action.

Since gene expression may be regulated partly by DNA methylation,[7] and since methylation inhibitors induce cellular differentiation,[8] we decided to limit our study to those agents that affect the pathway depicted in Fig 1. The two agents we chose were adenosine dialdehyde, a potent and specific S-adenosylhomocysteine hydrolase (EC 3.3.1.1, reaction 4, Fig 1) inhibitor,[9] and nitrous oxide (N_2O), an inhibitor of the cobalamin-dependent enzyme methionine synthetase (5-methyl-tetrahydrofolate homocysteine methyltransferase, EC 2.1.1.13, reaction 1, Fig 1).[10] We found that both agents induced HL-60 differentiation but that the differentiation appeared secondary to changes in methionine availability rather than to changes in transmethylation.

MATERIALS AND METHODS

Origin of cell line and culture conditions. The human promyelocytic cell line HL-60 was obtained from the American Type Culture Collection. The cells were routinely grown in RPMI 1640 medium (Irvine Scientific Co, Irvine, CA) supplemented with 2 mmol/L glutamine and 10% fetal bovine serum (FBS; Irvine Scientific Co) in a humidified atmosphere of 95% air/5% CO_2.

Measurement of HL-60 differentiation Differentiation of HL-60 cells along the myelocytic series requires approximately five to seven days. In fully differentiated HL-60 cells, as in normal granulocytes, membrane perturbation with phorbol-12-myristate-13-acetate (PMA) stimulates a number of biochemical reactions.[11] These reactions, summarized under the term "respiratory burst," include superoxide anion production and increased glucose oxidation via the pentose phosphate pathway The latter can be quantitated by measuring $^{14}CO_2$ release from [1-^{14}C]glucose [11,12] The ability of HL-60 cells to respond to PMA with increased $^{14}CO_2$ release from [1-^{14}C]glucose correlates with the proportion of mature cells in the population possessing a membrane-bound, PMA-stimulatable NADPH oxidase.[11] Respiratory burst activity can also be measured by counting the number of cells that reduce nitroblue tetrazolium.[4] We found that the two methods agreed well, but since the $^{14}CO_2$ release assay was more quantitative and less subjective, we have reported only these data.

For the induction of differentiation, cells were recovered by centrifugation from the growth medium and resuspended at a density of approximately 0.16 × 10^6 cells/mL in RPMI 1640 medium supplemented with 2 mmol/L glutamine and 10% horse serum (Irvine Scientific Co) that had been heat-inactivated at 56°C for 30 minutes and extensively dialyzed against 0.9% NaCl. The medium used in the experiments at low methionine concentrations was identical but contained methionine at 10 or 30 µmol/L instead of 100 µmol/L. After a 24-hour preincubation period in tissue culture flasks, 10 mL of the cell suspension was transferred to the wells of six-well cluster plates that contained the drug to be tested. In the N_2O experiments the cells were transferred to 25-mL culture flasks that were flushed with either 95% air/5% CO_2 or 75% N_2O/20% O_2/5% CO_2. Five days later cell density was determined by a Coulter Counter (Coulter Electronics, Hialeah, FL) and cell viability by trypan blue exclusion; only those cultures with greater than 90% trypan blue excluding cells were used.

For the determination of PMA-stimulated [1-^{14}C]glucose oxidation, cells were recovered by centrifugation and resuspended at a density of 1.5 × 10^6 cells/mL in glucose-free, bicarbonate-free, HEPES-buffered RPMI 1640 medium supplemented with 2 mmol/L glutamine and 0.2% dialyzed bovine serum albumin. Two 1-mL aliquots of the cell suspension from each of the duplicate cultures were transferred to 16 × 100-mm glass tubes; to one of the tubes PMA was added at a final concentration of 50 ng/mL After five minutes at 37°C in a shaking water bath, 0 4 µCi [1-^{14}C]glucose (final concentration 1 mmol/L; Amersham Radiochemicals, Arlington Heights, IL) was added, 20 minutes later the reaction was

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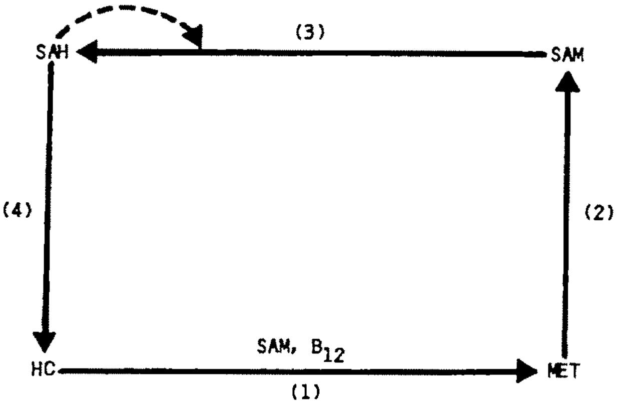

Fig 1. Methionine-homocysteine interconversion. Reactions: (1) methionine synthetase; (2) methionine adenosyltransferase; (3) methyltransferase reactions; and (4) S-adenosylhomocysteine hydrolase. HC, homocysteine; MET, methionine; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; and B_{12}, cobalamin. The broken arrow indicates that S-adenosylhomocysteine inhibits most methyltransferase reactions.

terminated by perchloric acid. $^{14}CO_2$ was collected and counted at approximately 90% efficiency as previously described[13] The assay was linear to 30 minutes and at cell densities between 0.5 to 2.5 × 10^6 cells/mL. The data are expressed as nanomole of $^{14}CO_2$ released per 30 minutes per 10^6 cells; PMA-stimulated [1-^{14}C]glucose oxidation was the difference in $^{14}CO_2$ release between the presence and absence of the PMA.

Measurement of S-adenosylmethionine and S-adenosylhomocysteine concentrations. Cells were recovered by centrifugation from the growth medium and resuspended at a density of 0.8 × 10^6 cells/mL in the horse serum-supplemented RPMI 1640 medium used in the differentiation studies After 20 hours the cells were harvested by pouring the suspension over an equal volume of frozen, crushed phosphate-buffered saline (PBS); this rapidly lowered the cell suspension temperature to approximately 6°C. The tubes were centrifuged at 4°C for three minutes at 800 × g, the cell pellet was extracted in perchloric acid and neutralized with KOH.

The nucleosides were measured by high-performance liquid chromatography on a Partisil SCX column (Whatman Inc, Clifton, NJ) in an isocratic system. For the S-adenosylhomocysteine 40 mmol/L $NH_4H_2PO_4$, pH 2 6, was used, and for the S-adenosylmethionine 0.4 mol/L $NH_4H_2PO_4$, pH 2 6, was used Peak areas at 254 nm were quantitated by comparison to standards obtained from Sigma Chemical Co, St. Louis; the concentration of both standards had to be corrected for the presence of small amounts of UV-absorbing contaminants.

RESULTS

Effect of adenosine dialdehyde and other S-adenosylhomocysteine hydrolase inhibitors on HL-60 growth and differentiation. Adenosine dialdehyde (2′-0-[(R)formyl-(adenine-9-yl)methyl]-(R)glyceraldehyde; 2′,3′-adenosine dialdehyde; periodate oxidized adenosine, Sigma #A7154, Sigma Chemical Co) induced HL-60 differentiation at concentrations of 1 μmol/L or greater; maximal differentiation occurred at 10 μmol/L (Table 1). Another S-adenosylhomocysteine hydrolase inhibitor, 2′-deoxyadenosine,[14] also induced HL-60 differentiation, but its toxicity, presumably through other mechanisms such as dATP accumulation,[15] limited its usefulness as an inducer. Two other S-adenosylhomocysteine hydrolase inhibitors, xylosyladenine and 5′-methylthioadenosine, also induced HL-60 differentiation (data not shown).

Table 1. Effect of Adenosine Dialdehyde, N_2O, or Cycloleucine on HL-60 Growth and Differentiation

Condition	Growth (Percent of Control)	PMA-Stimulated [1-^{14}C]Glucose Oxidation (nmol/30 min/10^6 cells)
Control	100%	0.41 ± 0.15
10 μmol/L Adenosine dialdehyde	31%	3.97 ± 0.91
+200 μmol/L Homocysteine thiolactone	94%	0.49 ± 0.18
75% N_2O	67%	2.57 ± 0 48
25 mmol/L Cycloleucine	26%	0.89 ± 0.21

Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated dialyzed horse serum. In the control condition the medium methionine concentration was 100 μmol/L with similar results at 30 μmol/L methionine, in the experiments with adenosine dialdehyde the medium methionine concentration was 100 μmol/L, while in the experiments with the N_2O and cycloleucine the medium methionine concentration was 30 μmol/L. After five days of culture $^{14}CO_2$ release from [1-^{14}C]glucose was measured, as described fully in "Materials and Methods." The values are the difference between the $^{14}CO_2$ release in the presence and absence of 50 ng/mL phorbol myristate acetate (PMA) and are the means ± SD of at least three experiments performed in duplicate.

Effect of adenosine dialdehyde on S-adenosylmethionine and S-adenosylhomocysteine concentrations in HL-60 cells. The normal ratio of S-adenosylmethionine to S-adenosylhomocysteine in HL-60 cells was 80.4 (Table 2). This ratio was markedly reduced to 3.3 by 0.1 μmol/L adenosine dialdehyde and further reduced to 1.6 by 10 μmol/L adenosine dialdehyde.

Induction of HL-60 differentiation by adenosine dialdehyde: Prevention by homocysteine. Since S-adenosylhomocysteine hydrolase inhibition by adenosine dialdehyde did not appear to induce HL-60 differentiation by altering the S-adenosylmethionine to S-adenosylhomocysteine ratio, we tested whether differentiation might be secondary to decreased homocysteine production. We found that the addition of 200 μmol/L L-homocysteine thiolactone to the culture medium completely prevented the induction of HL-60 differentiation by 10 μmol/L adenosine dialdehyde (Table 1).

Effect of N_2O on HL-60 growth and differentiation. Methionine synthetase requires methylcobalamin as a cofactor; the enzyme can be specifically inhibited by N_2O, which oxidizes the active cobalamin I to the inactive cobalamin II.[10] The other cobalamin-requiring enzyme methylmalonyl Co-A mutase (EC 5.4.99.2) uses adenosylcobalamin and is much less affected by N_2O.[16] As shown in Table 1, N_2O induced HL-60 differentiation. The degree of differentiation was inversely dependent on the methionine concentration in the medium; at 100 μmol/L methionine, N_2O was a poor inducer, but as the methionine concentration was

Table 2. Effect of Adenosine Dialdehyde and Cycloleucine on S-Adenosylmethionine and S-Adenosylhomocysteine Concentrations in HL-60 Cells

Condition	AdoMet (pmol/10^6 cells)	AdoHcy (pmol/10^6 cells)	AdoMet/AdoHcy
Control	217 ± 22.3	2.7 ± 0.5	80.4
0.1 μmol/L Adenosine dialdehyde	244 ± 31.2	74.5 ± 7.8	3.3
10 μmol/L Adenosine dialdehyde	318 ± 31.5	200 ± 18.4	1.6
10 mmol/L Cycloleucine	129 ± 23.8	2.0 ± 0.5	64.5

Cells were cultured in RPMI 1640 medium containing 100 μmol/L methionine supplemented with 10% heat-inactivated dialyzed horse serum. After 20 hours the cells were quickly harvested and extracted, as described in "Materials and Methods." The S-adenosylmethionine (AdoMet) and S-adenosylhomocysteine (AdoHcy) concentrations were measured by high-performance liquid chromatography and are the means ± SD of at least three experiments performed in duplicate.

decreased to the physiologic concentrations of 30 and 10 μmol/L, there was progressively more differentiation (the data shown are for 30 μmol/L methionine).

Effect of cycloleucine on the growth, differentiation, and S-adenosylmethionine concentration of HL-60 cells. Since decreased methionine availability could also decrease S-adenosylmethionine synthesis, cells were treated with cycloleucine, a relatively specific methionine adenosyltransferase (EC 2.5.1.6) inhibitor.[17] We showed that at 10 mmol/L cycloleucine inhibited methionine adenosyltransferase (reaction 2, Fig 1) activity in cell extracts by more than 90% and significantly decreased the intracellular S-adenosylmethionine concentration (Table 2). In concentrations as high as 24 mmol/L this drug did not induce HL-60 differentiation at methionine concentrations of 10 to 100 μmol/L in the medium (Table 1).

DISCUSSION

These studies demonstrate that adenosine dialdehyde and N_2O, highly specific inhibitors of S-adenosylhomocysteine hydrolase and methionine synthetase, respectively, induce HL-60 differentiation. Adenosine dialdehyde is not known to affect other reactions, and we have shown that at micromolar concentrations it is not phosphorylated to a nucleotide.[18] In cultured cells the primary effect of N_2O is methionine synthetase inhibition.[19]

Our initial hypothesis was that these two agents were operating by decreasing rates of methylation: adenosine dialdehyde through S-adenosylhomocysteine accumulation leading to inhibition of methyltransferase reactions (reaction 3, Fig 1) and N_2O through decreased S-adenosylmethionine synthesis because of decreased methionine synthesis. The subsequent work did not support this hypothesis, however. In the case of adenosine dialdehyde, there was a lack of correlation between drug concentrations that changed the S-adenosylmethionine to S-adenosylhomocysteine ratio and drug concentrations that induced differentiation. It is, of course, possible that a threshold effect exists and that the further small reduction in the S-adenosylmethionine to S-adenosylhomocysteine ratio produced by 10 μmol/L, as compared with 0.1 μmol/L, adenosine dialdehyde caused major changes in transmethylation. The complete prevention of induction of differentiation by homocysteine strongly suggested that it was a homocysteine deficiency, rather than a S-adenosylhomocysteine excess, that was responsible for the induction of differentiation. The lack of induction of differentiation by cycloleucine at concentrations that significantly reduced the S-adenosylmethionine concentration suggested that N_2O's effect was not mediated through decreased S-adenosylmethionine synthesis. It is of interest that recent data from other systems suggest that changes in gene methylation are only of minor importance during differentiation.[20]

There are at least two other mechanisms to explain the induction of HL-60 differentiation by adenosine dialdehyde and N_2O. The first is that these agents decrease methionine availability, and the second is that they interfere with folate metabolism. In cultured lymphoblasts approximately 20% of the intracellular methionine is used in the reactions shown in Fig 1.[21] Interruption of this cycle by either adenosine dialdehyde or N_2O will lead to intracellular accumulation and extracellular excretion of S-adenosylhomocysteine or homocysteine, respectively, thereby draining the cell of methionine.[22,23] This mechanism is supported by the homocysteine prevention of the adenosine dialdehyde-induced differentiation and by the increasing effectiveness of N_2O as an inducer at lower methionine concentrations. We have previously shown in cultured lymphoblasts that both adenosine dialdehyde and N_2O appear to cause trapping of 5-methyltetrahydrofolate.[18,19] 5-methyltetrahydrofolate is a major storage form of intracellular folates, and its synthesis from 5,10-methylenetetrahydrofolate is essentially irreversible in vivo.[24] Conversion of 5-methyltetrahydrofolate to free tetrahydrofolate requires an adequate supply of homocysteine in addition to intact methionine synthetase activity (cobalamin and S-adenosylmethionine are required as enzyme cofactors; Fig 1). Support for this mechanism, that decreased folate availability leads to differentiation, is that methotrexate is a potent, albeit very toxic, inducer of HL-60 differentiation.[25] Because folate (pteroylglutamic acid) was present at 2 μmol/L in the medium and addition of up to 1 mmol/L folate did not prevent induction of differentiation by either the adenosine dialdehyde or the N_2O (data not shown), decreased folate availability seems less likely to be a mechanism of induction of differentiation in our studies than decreased methionine availability.

Decreased methionine availability could lead to HL-60 differentiation through at least two different mechanisms. First, it could decrease purine nucleotide synthesis. Methionine is a major intracellular source of formate, and through folate-dependent reactions formate is a precursor of the purine ring;[24,26,27] in addition, essential amino acid starvation leads to decreased purine nucleotide synthesis.[28] Several agents that inhibit purine synthesis, such as 6-mercaptopurine, 6-thioguanine, and virazole, induce HL-60 differentiation.[4,25] Second, methionine limitation can decrease protein synthesis initiation, since mRNA translation always begins with a methionyl codon. Decreasing rates of protein synthesis initiation can change which mRNAs are preferentially translated;[29] mRNA discrimination is one form of control of gene expression at the translational level and may be operative during cellular differentiation.[30] Future studies will be aimed at determining the mechanism(s) of induction of differentiation by methionine limitation and whether starvation for other essential amino acids also induces HL-60 differentiation.

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Relationship Between T200 Antigen Expression and Stages of B Cell Differentiation in Resurgent Hyperplasia of Bone Marrow

By Charles W. Caldwell and William P. Patterson

Using monoclonal antibodies (MoAbs) and dual-parameter flow cytometric techniques, bone marrow mononuclear cells (MMC) from patients with resurgent hyperplasia were analyzed for their coexpression of HLe-1 (T200) and antigens normally associated with particular stages of B cell differentiation. The marrow from those with resurgent hyperplasia contained increased numbers of B cell precursors in multiple stages of differentiation compared to controls, thus providing a useful model system for studies of B cell differentiation. These studies indicate that the quantitative expression of T200 is differentiation-related on normal and malignant B cells and B cell precursors. Immature cells express low amounts of T200, while increasing levels of maturity correlated with increasing amounts of the antigen. This study increases the understanding of relationships between B cell surface antigens and T200 and further demonstrates that B cell hyperplasia occurs commonly in association with bone marrow reactive or resurgent processes. The quantitative, rather than only the qualitative, expression of T200 is therefore a useful marker of B cell differentiation in reactive hyperplasia and in further investigation of B cell malignancy.

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PREVIOUS STUDIES have shown that lymphoblasts of patients with acute lymphoblastic leukemia (ALL) as well as cells of patients with other lymphoid malignancies exhibit decreased expression of HLe-1, an epitope of the T200 antigen.[1-3] During these studies it was noted that in some marrow specimens following chemotherapy and in marrow specimens of patients with nonmalignant reactive processes, dim fluorescence peaks were present in addition to the normally bright peaks after staining with anti-HLe-1 (T200) monoclonal antibody (MoAb). In many of the cases of ALL in which these peaks were seen, the patients entered complete remission after therapy. These observations raised the possibility that these dim peaks of T200 were associated with resurgence of normal, early B cells in bone marrow recovering from therapy. Since B cell precursors originate from the bone marrow,[4-8] it is not surprising that multiple subpopulations might be demonstrable in this tissue. This paper represents an attempt to evaluate this possibility by analysis of bone marrow mononuclear cells (MMC) for their coexpression of HLe-1 (T200) and antigens normally associated with particular stages of B cell differentiation.[9,10]

MATERIALS AND METHODS

Study population. Bone marrow was obtained from children (ages 3 to 15) undergoing diagnostic evaluations for various suspected or documented disease processes. These included two patients (each on two separate occasions) with cyclic neutropenia, one following chemotherapy for a T cell ALL, two with immune thrombocytopenia, three with neuroblastoma, one with Ewing's sarcoma, and one with hepatitis B. Tumor was not found in any of the bone marrow specimens at the time of study. Bone marrow aspirates used as controls were obtained from children (ages 4 to 12) undergoing routine clinical monitoring for previously diagnosed and treated ALL (n = 10). All these children were receiving maintenance chemotherapy, and all were considered to be in remission of their leukemia at the time of examination and on follow-up 6 months later. Peripheral blood was collected from healthy individuals aged 2 to 20 years (n = 50). Specimens were collected after informed consent in accordance with guidelines established by the Institutional Review Board of the University of Missouri School of Medicine.

Preparation of specimens for analysis. Peripheral blood and bone marrow were aseptically collected by standard techniques. The peripheral blood lymphocytes (PBL) and MMC were enriched by density gradient centrifugation as previously described.[2,11] PBL and MMC were adjusted to 1 × 10^7 cells/mL in RPMI 1640 medium and analyzed within two hours of receipt. Bone marrow smears from aspirated cells were visually examined after staining with May-Grünwald Giemsa. The marrow was also examined for the presence or absence of malignancy in fixed tissue preparations of clotted marrow aspirate or needle-biopsy core sections.

MoAbs and immunostaining. The specificities and sources of MoAbs used in this study are listed in Table 1. Several of these MoAbs were available as both fluorescein isothiocyanate(FITC)- and phycoerythrin(PE)-labeled reagents. The anti-immunoglobulin reagents and anti-T200 MoAbs were unconjugated and therefore used in indirect methods of staining. The MoAb J-5 (Coulter Electronics, Hialeah, FL) was labeled with RD-1, which is similar to PE in its excitation and emission spectra. A panel of antimyeloid MoAbs was also used in some cases to determine the level of myeloid contamination of the light scatter peaks (data not shown).

Aliquots (50 μL) of the cell suspensions (1 × 10^7 cells/mL) and 50 μL of FITC-labeled MoAbs (at saturating concentrations) were incubated at 4°C for 30 minutes and the cells analyzed with the flow cytometer. A monoclonal FITC-labeled isotypic negative control was analyzed in parallel with each specimen. Previous work has shown the reproducibility and stability of this "no-wash" staining procedure.[11,12]

For dual-parameter staining, aliquots of 50 μL of cell suspension were added to 50 μL of FITC-labeled MoAb and 50 μL of PE (or RD-1)-labeled MoAb. After incubation at 4°C for 30 minutes, the cell suspension was washed twice with cold phosphate-buffered saline (PBS)/azide, resuspended in 0.5 mL of cold buffer, and submitted to flow cytometric analysis.

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Table 1. Monoclonal Antibodies Used in This Study

MoAb	Specificity	Source
HLA-DR	Nonpolymorphic HLA-DR epitopes. Present on all early B cells and lost at plasma-cell stage.	B
Leu-12	Appears earlier than Leu-16 on pre-B cells and is lost early in plasma-cell differentiation (CD 19)	B
CALLA	Common acute lymphoblastic leukemia antigen, present on early pre-B cells, lost on sIg$^+$ cells (CD 10)	B
J-5	Anti-CALLA (CD 10)	C
Leu-16	Appears on early pre-B cells and is lost at plasma-cell stage (CD 20)	B
B2	CR 3, mature B cells (CD 21)	C
IgM	Mu heavy chains	C
IgG	Gamma heavy chains	C
Kappa	Kappa light chains	C
Lambda	Lambda light chains	C
OKT11	E-rosette receptor on T cells (CD 2)	O
IL 2R	Interleukin 2 receptor (CD 25)	B
	Anti-T200 Antibodies	
HLe-1	B and T cells, granulocytes, monocytes (CD 45) (all T200 bands)	B*
T29/33	Strong expression on B cells (gp 220) and T cells (gp 200)	M
F8-11-13	Strong staining of B cells, weak staining of a small subpopulation of thymocytes and T cells (gp 215)	†
F10-89-4	B cells stain weaker than T cells (gp 190-215)	†
G1-14.2	B and T cells, all four major gp bands (190-220)	‡
G25-1	Similar to G14 in pattern of reactivity, but marks different epitope	‡

Abbreviations: CD, Clusters of differentiation, as defined by the Second International Workshop on Human Leukocyte Differentiation Antigens[27,28]; C, Coulter Immunology, Hialeah, FL, O, Ortho Diagnostic, Raritan, NJ, B, Becton-Dickinson, Mountain View, CA; M, Boehringer-Mannheim, Indianapolis.

*Biotinylated HLe-1 was a generous gift of Becton-Dickinson, Mountain View, CA

†These were generous gifts of Dr R Dalchau, Sussex, UK [24,25]

‡These were generous gifts of Dr J Ledbetter, Seattle.[26]

The anti-T200 MoAbs were used in an indirect method of immunostaining. Aliquots (50 μL) of cells were first incubated with saturating concentrations of each unlabeled MoAb, washed twice in PBS, and aliquots (100 μL) of FITC-labeled sheep antimouse immunoglobulin (Fab$'_2$ fragments) were added at saturating concentrations. After a second 30-minute incubation at 4°C, the cells were again washed twice in PBS and submitted to flow cytometric analysis.

Flow cytometric analysis. An EPICS V flow cytometer (Coulter Electronics) was operated at approximately 500 mW in the 488-nm line. The FITC signal was filtered through a 515-nm long-pass filter. Standardization of forward-angle light scatter (FALS) and log integral green fluorescence (LIGFL) signals were performed as described.[11,12]

For demonstrations of antigen coexpression, cells were analyzed by passing the fluorescence signal through a 560-nm dichroic mirror to split the beam for red fluorescence (collected through a 570-nm long-pass filter) and green fluorescence (collected through a 525 ± 10-nm band-pass filter). The green and red fluorescence subtraction modules were set to eliminate spectral crossover of FITC- and PE-MoAb positive cells into the photomultiplier tube (PMT).

Single (and dual) parameter histograms were analyzed for percentages of positive cells using computer-assisted programs (INTEGRATE, IMMUNO, STATS; Coulter). Fluorescence intensity was determined from single- or dual-parameter histograms with computer assistance (STATS; Coulter).

Immunoalkaline phosphatase staining for cytoplasmic Mu heavy chains. The percentage of cells expressing cytoplasmic IgM heavy chains (cIgM) was determined by immunostaining with MoAb against human IgM with the cells in suspension (surface IgM) and after fixation on slides (cIgM). Monoclonal anti-IgM (Coulter) was used with a commercial immunoalkaline phosphatase kit (Vectastain, Burlingame, CA).

Immunoadsorption of cell populations. In some experiments certain cell populations were removed from the specimen by "panning" prior to analysis for HLe-1 and other markers. The panning procedure was performed as described.[13]

RESULTS

Bone marrow morphological changes. Morphological evaluation of resurgent bone marrow specimens revealed various patterns, some with obvious mononuclear hyperplasia and others with nonspecific changes. Although the percentage of blasts was not increased in all specimens, there was usually an increase in lymphocytes, some mature and others less mature, having diffuse nuclear chromatin, scant cytoplasm, and indistinct nucleoli. Many of these lymphocytes are actually B cell precursors.[14]

Single MoAb positivity. The overall pattern of immunostaining in resurgent marrows suggests a "left shift" toward immature B cells and is similar to the pattern in fetal bone marrow,[9] while control marrows are similar to adult patterns.[5] There is a difference in the percentages of B cell precursor when resurgent marrows are compared with controls (Table 2). Control pediatric marrow contains mainly T lymphocytes (OKT11) with low numbers (<10%) of B cells and B cell precursors. The percentage of HLA-DR positivity is increased over the percentage of positivity with pan-B cell markers (Leu-12 and Leu-16) and is related to contamina-

Table 2. Percentages of MoAb-Defined Subsets in Control and Resurgent Bone Marrow

MoAb	Control Marrow (n = 10)	Resurgent Marrow (n = 10)
HLA-DR	*23.7 ± 6.7	67.7 ± 9.7
Leu-12 (CD 19)	8.1 ± 3.7	45.8 ± 9.1
CALLA (CD 10)	4.0 ± 2.5	48.3 ± 14.3
Leu-16 (CD 20)	6.9 ± 3.9	27.5 ± 14.5
cIgM†	4.9 ± 4.1	10.4 ± 9.1
B2 (CD 21)	6.3 ± 3.4	7.3 ± 2.4
sIg	5.1 ± 3.9	5.9 ± 4.1
OKT11 (CD 2)	73.1 ± 17.0	44.4 ± 15.6
IL 2R (CD 25)	3.0 ± 2.4	6.4 ± 5.1

*Numbers represent the mean percentage (±SD) of MoAb-positive cells from within the lymphoid light scatter gates.

†Percentages of cells positive for cIgM were determined from immunoalkaline phosphatase (IAP) staining. Values represent the percentage of mononuclear cells after correction for myeloid contamination as determined using an antimyeloid cocktail in the IAP staining procedure.

tion with a few erythroid and myeloid cells (data not shown) and possibly early Leu 12^- B cell precursors.[9,10] Most of the B cells in control pediatric marrow are relatively mature, as demonstrated by the similarity in percentage of positivity with B2, Leu-16, and sIg. A small percentage (<5%) of cells are positive for common acute lymphoblastic leukemia antigen (CALLA) and cIgM, indicating small numbers of early B cells. Additionally, a few (3%) of the cells are positive for the IL 2R, suggesting at least a low level of T or B cell activation. These values are similar to those reported in normal adult marrow.[5,9]

In resurgent marrow, lower percentages of T cells and higher percentages of B cell precursors are present (Table 2). The HLA-DR positivity still exceeds the percentage of Leu 12^+ cells. Compared to control marrow, marked increases are seen in cIgM$^+$, HLA-DR$^+$, Leu-12^+, CALLA$^+$, and Leu-16^+ cells. These MoAbs are present on B cell precursors. Percentages of B2$^+$ and sIg$^+$ cells remain similar to controls, thus the majority of B cells present in resurgent marrow are at immature stages of development. A mild increase is also seen in IL 2R positivity, consistent with a modest degree of cellular activation. Dual marking of these cells demonstrated both IL 2R-positive T cells and B cells (data not shown).

Fluorescence intensity of single MoAb. Differences in the FI of cells from peripheral blood, control pediatric marrow, and resurgent marrow are apparent (Table 3 and Fig 1). In peripheral blood, which contains only mature B cells, a single peak of FI was present with each MoAb. In control marrow, single peaks were also found, with the exception of HLe-1. A few (three of ten) of the specimens from children with durable remissions of ALL and recovering from maintenance chemotherapy had small secondary peaks (<5% of cells) of dim HLe-1 FI that represented low numbers of B cell precursors. In all three of these cases, the FI of the secondary peak was significantly different than that of their ALL blasts at diagnosis. This type of HLe-1 staining has been found in the course of monitoring children during maintenance chemotherapy (data not shown). Nonmalignant marrow specimens from children and adults evaluated for iron deficiency anemia contain only one HLe-1 peak of FI (data not shown).

Resurgent specimens contained more than one peak of FI with all MoAbs except Leu-12, thus suggesting the presence of multiple subpopulations of cells. Although Leu-12 produced only one peak, it was dimmer than that observed in control marrow and peripheral blood. In the case of HLA-DR, the major peak was bright and corresponded to that seen in control marrow and blood. The dim HLA-DR peak consisted of T cells and immature B cells by dual marker studies (data not shown). The peaks of FI with CALLA were of a similar pattern in all resurgent specimens and differed from controls in that neither of the two peaks corresponded to the CALLA FI of control specimens. This is probably due to the low number of both cell types (dim and bright) that blend into a single histogram of average intermediate FI in control specimens. The major peak of CALLA in resurgent marrows was the dimmest, and the bright peak (not present in normal marrows) was a lower percentage of cells. Staining with Leu-16 also produced two peaks, a bright peak similar to that in control marrow and blood and a dim peak not seen in these specimens. The MoAb HLe-1 usually produced three distinctive FI peaks (and occasionally four) in the resurgent specimens. The brightest of these was similar to that of mature peripheral blood B and T lymphocytes, while the dimmer peaks were not seen in blood. An intermediate peak corresponded in FI to the small peak seen in some control marrow specimens, but the dimmest peak was not present in controls. Therefore not only are the number of FI peaks different from normal but also the quantitative FI of the peaks.

Relationship of HLe-1 to B cell-associated MoAbs. Figure 2 illustrates the relationship of HLe-1 (T200) FI to that of other B cell-associated (or T cell) MoAbs on subpopulations of cells from a representative resurgent specimen. In each histogram, regions identified as 1 to 4 are associated with increasing FI of HLe-1-stained cells. Multiple peaks of increasing HLe-1 FI (y-axis) are present from cells within the lymphoid region by FALS (x-axis). Cells from this

Table 3. Fluorescence Intensity of Single MoAb-Stained Bone Marrow Cells

MoAb	PB (n = 50) Peak 1*	Control Marrow (n = 10) Peak 1	Control Marrow (n = 10) Peak 2	Resurgent Marrow (n = 10) Peak 1	Resurgent Marrow (n = 10) Peak 2	Resurgent Marrow (n = 10) Peak 3
HLA-DR	100 ± 10†	93 ± 11		102 ± 8	24 ± 4	
	(14 86)‡	(12.30)		(1.91)	(15.68)	
Leu-12	56 ± 7	47 ± 9		34 ± 3		
	(4.53)	(3.56)		(2.50)		
CALLA	NA	53 ± 9		40 ± 5	78 ± 6	
	(NA)	(4.18)		(2.94)	(8.20)	
Leu-16	82 ± 19	80 ± 12		92 ± 17	29 ± 8	
	(9 14)	(8 66)		(11.97)	(2.19)	
HLe-1	126 ± 4	129 ± 4	76 ± 8§	127 + 6	80 ± 5	29 ± 9
	(29 96)	(32.49)	(7.77)	(30.78)	(8.66)	(2.19)

*Peaks 1, 2, and 3 represent separate but sometimes overlapping subsets of cells within each histogram In all cases peak 1 is the main peak in each histogram, followed in decreasing order by peaks 2 and 3

†The mean channel of fluorescence intensity ± the SD is expressed on a linear scale (256 channels) after logarithmic amplification and thus represents the geometric mean of fluorescence intensity.

‡Numbers in parentheses represent the mean fluorescence intensity expressed as linear arbitrary fluorescence units (see Materials and Methods)

§Three of the ten control specimens contain this intermediate FI peak of HLe-1. In all three this peak was 5% of the total MoAb-positive cells

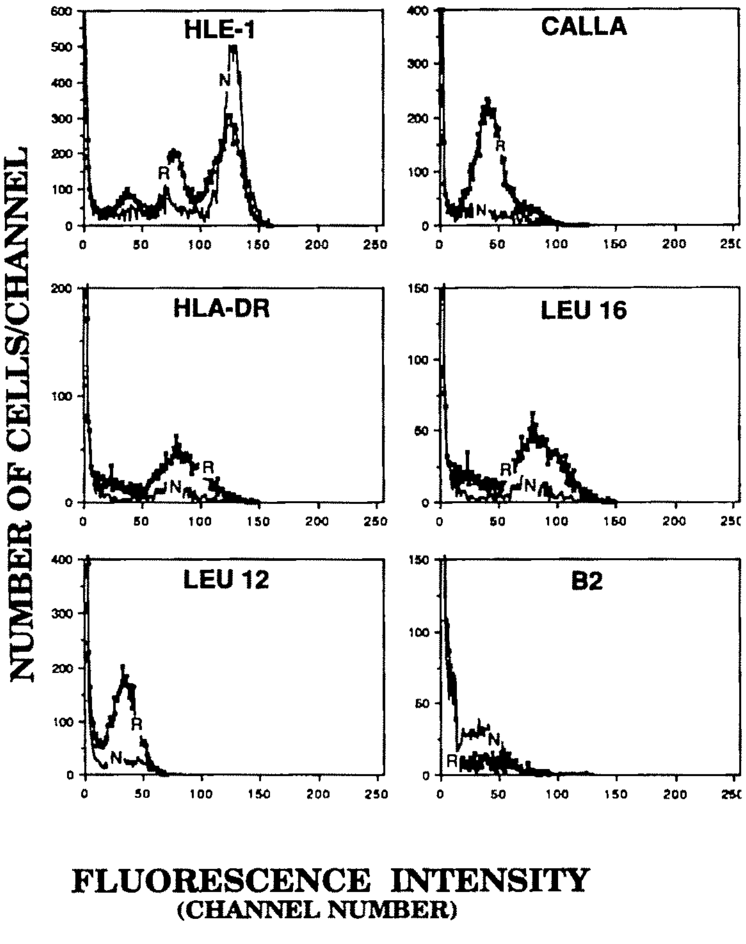

Fig 1. Comparison of MoAb-defined histograms of T200 and B cell-associated antigens between control (N) and resurgent (R) bone marrow. Directly labeled MoAbs and mononuclear cells from representative marrow aspirates were used to construct fluorescence histograms of 10,000 cells (HLe-1 contains 20,000). The resurgent and normal histograms with each MoAb are illustrated at the same scale factor. Fluorescence intensity is the logarithmically amplified signals plotted on a linear scale.

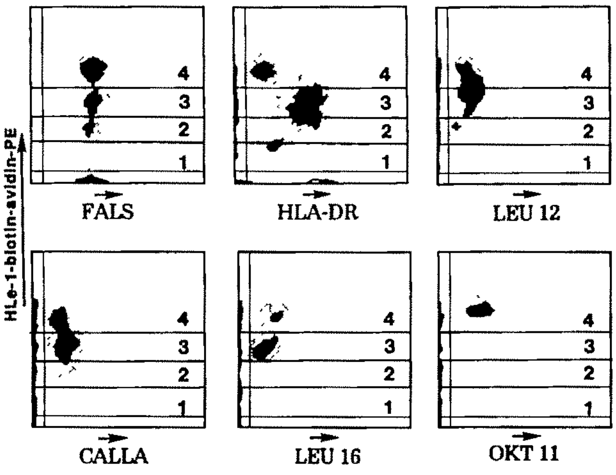

Fig 2. Dual-parameter histograms of a representative resurgent bone marrow immunostained with HLe-1–biotin-avidin PE (y-axis) and each of the indicated FITC-labeled MoAbs or forward-angle light scatter (x-axis). All histograms contain 20,000 cells. Fluorescence is illustrated after 3 decade logarithmic amplification. Cells negative for either parameter cluster on the baseline at the gain settings employed (horizontal and vertical light lines). Regions 1 to 4, delineated by heavy lines in each histogram, are shown to identify similar FI with HLe-1.

specimen were also simultaneously immunostained with HLe-1 (y-axis) and each of the indicated FITC-labeled MoAbs (x-axis).

Simultaneous staining with HLA-DR produced three dual-stained peaks as well as a small population of HLA-DR$^+$/HLe-1$^-$ cells and cells that were HLA-DR$^-$/HLe-1$^+$. Dual staining with a cocktail of antimyeloid MoAbs (My4, MY7, MY9; Coulter Immunology) and HLA-DR produced 4% dual-stained cells; therefore only a small part of these were myeloid precursors (data not shown). The HLA-DR$^-$/HLe-1$^+$ cells were found to be T cells on dual-staining with OKT11 (not shown). Of the three peaks of dual-positive cells, those in region 1-2 were simultaneously dim for both, while region 3 cells were bright for HLA-DR and spanned the region of dim–intermediate HLe-1 FI. Region 4 contained mainly "activated" T cells expressing low levels of HLA-DR and a few mature B cells with bright HLA-DR. This histogram demonstrates that as HLe-1 FI increases, HLA-DR FI increases on B cells.

Three dual-stained peaks of Leu-12 and HLe-1 are present. Of these the Leu-12 FI is very dim on cells in region 2, increases to a maximum on the major HLe-1 peak (region 3), and then decreases slightly in Leu-12 FI on HLe-1 bright cells (region 4). Leu-12 first appears on cells that are somewhat brighter for HLe-1 than does HLA-DR, suggesting the initial appearance of Leu-12 on more mature cells than HLA-DR.

Simultaneous staining with CALLA and HLe-1 produced two peaks of dual positivity. The brighter CALLA FI was associated with intermediate HLe-1 FI (region 3), while dimmer CALLA FI was associated with brighter HLe-1 FI (region 4). Therefore, as previously reported, CALLA FI decreases with maturity.[15,16]

Leu-16 was dimly positive on intermediate HLe-1 FI cells (region 3) and at its maximum FI on HLe-1 bright cells. This is opposite the pattern seen with CALLA. Simultaneous staining for kappa and lambda light chains and HLe-1 demonstrated all the dual positivity to be associated with bright HLe-1 (not shown). As illustrated, OKT11$^+$ cells are contained mainly in the brightest HLe-1 peak (region 4), with fewer (and dimmer) OKT11$^+$ cells in the intermediate HLe-1 peak (region 3). Although every specimen did not exhibit the same percentages of precursor cells, all resurgent marrows demonstrated the same relationships of FI between MoAbs. A small number of erythroid precursors were also found in the HLe-1 dim peak (data not shown). The sequential initial appearance (from HLe-1 dim to HLe-1 bright) of HLA-DR, Leu-12, CALLA, and Leu-16 suggests a direct relationship between the level of HLe-1 FI and increasing stages of B cell differentiation.

HLe-1 FI and cIgM after selective cell depletion. Figure 3 illustrates the HLe-1 FI histograms of MMC before and after selective removal of specific cellular subsets by immunoadsorption ("panning"). In addition to HLe-1 FI, percentages of cIgM$^+$ cells were calculated on each sample undergoing MoAb-defined subset removal. The top histogram (NONE) represents the HLe-1 FI peaks from a sample of resurgent marrow in which no subsets were removed Three discreet peaks of HLe-1 FI are present, and on this basis the histogram was divided into four regions. Selective depletion of cells by various MoAbs or combinations of MoAbs produced losses of HLe-1$^+$ cells from particular regions of the histogram. The percentage of HLe-1$^+$ cells remaining in each region after panning are indicated for each histogram.

In the histogram labeled "Mature Panel," OKT11$^+$, Leu-2$^+$, Leu-3$^+$, and sIg$^+$ cells were removed. This reduced the percentage of HLe-1$^+$ cells with the highest FI to 31% and increased the relative numbers of less bright HLe-1$^+$ cells as well as cIgM$^+$ cells. This demonstrates that losses of the more mature cells lead to a loss of cells with the brightest HLe-1 FI.

By using immunoadsorption directed at less mature cells (Leu-12, B1 [Leu-16], and CALLA), decreased percentages were seen in the regions of dimmer HLe-1 FI. In addition, the percentages of cIgM$^+$ cells were decreased by 43% to 68%. The histograms labeled Leu-12, B1, and CALLA show an increase in percentages of the brightest HLe-1$^+$ cells as compared to the "Mature Panel" and the "None" histogram, with a decrease in numbers of dimmer cells in the histograms in which less mature cells were removed.

Relationships of FI in dual-stained populations. Based on the above observation that resurgent marrow contained multiple subpopulations of B cell precursors, additional dual-staining was done to determine the relationships of the FI of these MoAb-stained cell populations to each other. In this way it may be possible to define the sequence of quantitative antigen expression as a function of cell differentiation. Data from a representative resurgent marrow specimen (cyclic neutropenia) are presented in Fig 4. All the resurgent specimens produced similar patterns but different percentages of cells in each peak.

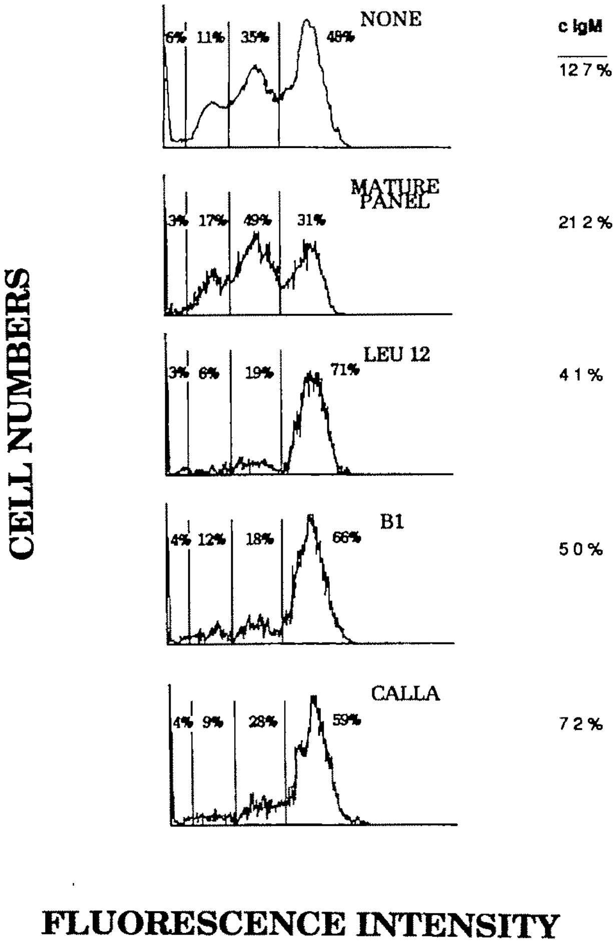

Fig 3. Fluorescence histograms of HLe-1-stained cells from resurgent bone marrow before (NONE) or after panning with a "Mature Panel" (consisting of OKT11, Leu-2, Leu-3, kappa, lambda), Leu-12, B1, or CALLA. Percentages of cells in each of four regions were calculated based on channel numbers of the non-panned specimen (NONE). The percentage of cIgM$^+$ cells before and after panning with each MoAb or panel is also presented.

HLe-1-stained cells *v* FALS (cell size) demonstrates five peaks of differing FI. Peaks 1 to 4 are cells from within the "lymphoid" area, while peak 5 contains myeloid cells Cells from peak 5 were not included in the remaining histograms. Peak 1 is contaminating RBC; peak 2 contains a few erythroid precursors but mainly Leu 12$^+$ B cells; and peaks 3 and 4 contain lymphoid cells.

Four peaks of Leu 12$^+$ cells are discernable after dual staining with HLe-1. Peak 1 contains few cells and is negative for HLe-1 but dimly positive for Leu-12. About half

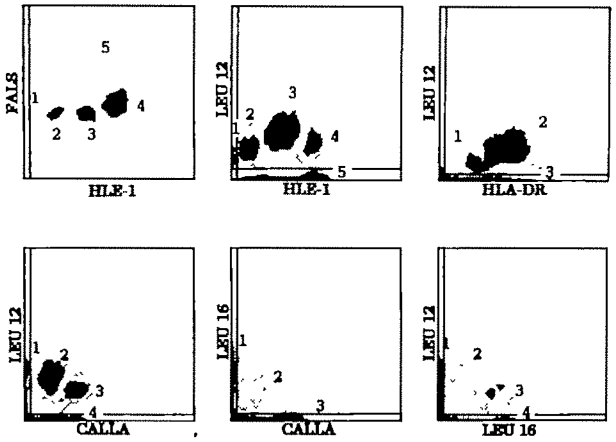

Fig 4. Dual-parameter analysis of immunostained cells for coexpression of T200 and B cell-associated surface antigens. On the y-axis cells were examined for forward-angle light scatter (FALS) or fluorescence after staining with each PE-labeled MoAb, as indicated. Cells were simultaneously immunostained with FITC-labeled MoAbs (x-axis). Each histogram is composed of 20,000 data points. Vertical and horizontal lines indicate cut-off levels for positivity based on isotypic negative control MoAbs (data not shown). The numbers indicate separate subpopulations of immunostained cells in each histogram.

the resurgent specimens contained a few of these cells, thus it is not entirely clear which antigen is expressed first. Peak 2 is only slightly brighter than peak 1 in terms of Leu-12 FI, while the HLe-1 FI is clearly brighter. A few HLe-1^+/Leu-12^- cells (erythroid precursors) are also found within this dim HLe-1 peak (data not shown). The third peak shows the brightest Leu-12 FI and intermediate HLe-1 FI. Panning experiments (Fig 3) indicate these are enriched in cIgM^+ and cIgM^- pre-B cells. A small number of T cells (OKT11^+) are also in peak 3. Peak 4 contains mature sIg^+ B cells that clearly have the brightest HLe-1 FI but now have Leu-12 FI that has decreased somewhat to a level intermediate between that of peaks 2 and 3. The level of HLe-1 FI in peak 4 is slightly less than that for T cells in most specimens examined.

Dual staining with HLA-DR and Leu-12 shows that all Leu-12^+ cells are simultaneously HLA-DR^+ and that although two separate peaks of FI are present, there is a direct correlation in FI. The cells in peak 1 are dim for both MoAbs, while the cells in peak 2 stain brighter with both. The cells in peak 3 (extending along the baseline of Leu-12) may represent very immature B cells, activated T cells, myeloid blasts, or any combination of these possibilities. A small percentage of each of these cell types was found by dual staining (data not shown).

Dual staining with CALLA and Leu-12 produced two dual-stained populations of cells, one of which is brightly positive for Leu-12 but dim for CALLA (peak 2) and the other of which contains cells that are dim for Leu-12 but bright for CALLA (peak 3), suggestive of an even less mature cell population than peak 2.[15,16]

Dual staining with CALLA and Leu-16 shows that most of the Leu-16^+ cells are CALLA^- (peak 1). Similarly, most of the CALLA^+ cells are Leu-16^- (peak 3). Only a small population of dual-stained cells is present (peak 2). These are dim for both antigens, suggesting CALLA^+ precursors at a later stage of differentiation than most of those found with Leu-12 and CALLA. Based on their very dim CALLA FI, these cells probably represent the most mature subpopulation of CALLA^+ cells.

Dual staining with Leu-16 and Leu-12, both overlapping markers of B cell subsets, shows that of the four populations present, most are Leu-12^+/Leu-16^- (peak 1), and a small number are Leu-12^-/Leu-16^+ (peak 4), suggestive of plasmacytoid B cells. Peak 2 contains a small (2%) population of Leu-12–bright, Leu-16–dim cells, and peak 3 contains cells that are brightly positive for Leu-16 but of intermediate FI for Leu-12.

Overall the cross-correlations of FI with these combinations of MoAbs support reported schemes of B cell differentiation[9,10,15,16] and relate these to HLe-1 FI (Figs 2 and 4).

Additional Anti-T200 MoAb. Figure 5 shows the pattern of FI in a reactive marrow using a panel of anti-T200 MoAb that identifies various epitopes of the antigen. This panel was used to determine if different epitopes of the T200 antigen are expressed at different times during cell differentiation or if all are expressed in a similar pattern. In general, the MoAbs T29/33, F10-89-4, G1-14.2, and G25-1 all show multiple peaks of FI similar to those observed with HLe-1. However, the histograms of some of these MoAbs show additional subpopulations or shifts in the distribution of cells

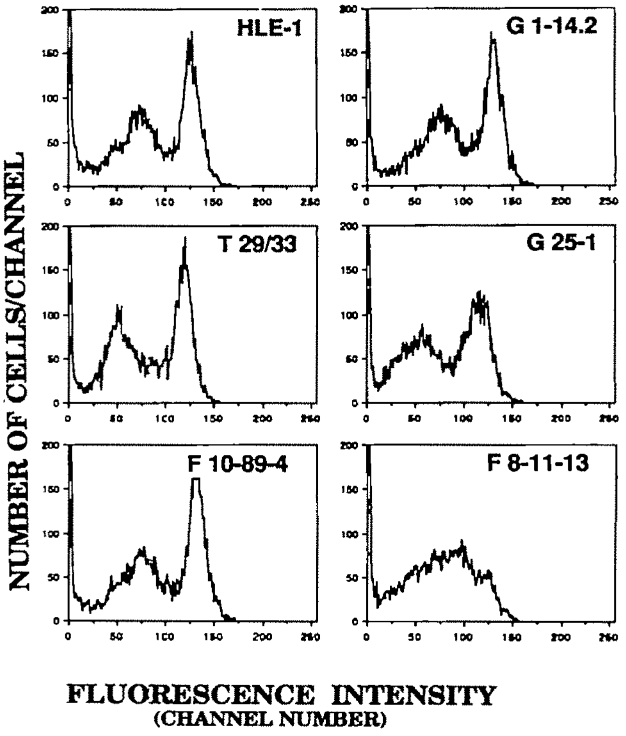

Fig 5. Comparison of fluorescence histograms for a representative resurgent marrow immunostained with each of six MoAbs that identify various epitopes of the T200 antigen. Each histogram contains 10,000 cells from within the lymphoid light scatter gates. All histograms are illustrated at the same scale factor. Fluorescence intensity is illustrated on a linear scale after logarithmic amplification.

within the histograms. It is possible that not all these epitopes are quantitatively expressed on the same cells at the same time during differentiation. In general, though, all these MoAbs appear to define immature B cells as decreased FI. F8-11-13-stained cells show a rather broad spectrum of FI. In part this is because many of the T cells are negative for this MoAb, which is restricted in its specificity (Table 1). While the brightest fluorescent peak with each MoAb remains in a similar position (~channel 120), the intermediate and dim cells shift in their relative FI with different MoAbs. Overall it appears that quantitatively the T200 epitopes identified by the FI of these MoAbs are all expressed in a differentiation-related manner, dim on immature cells and brighter on more mature cells, but not all are necessarily expressed quantitatively at the same time during B cell differentiation.

DISCUSSION

Bone marrow B cell precursors have been shown to be increased in several disease states associated with reactive marrow hyperplasia. These cells conform immunophenotypically to the subpopulations of precursor B cells in bone marrow previously described,[9,10,15,16] thus providing an excellent model of in vitro B cell differentiation. In vitro studies by Nadler et al[9] revealed at least four subsets of B cell precursors in normal marrow. Similarly, Hokland et al[16] demonstrated an orderly expression of B cell antigens in fetal bone marrow stimulated to differentiate in vitro. This study confirms the in vitro work by Hokland et al[16] and demonstrates the progressive increase in quantitative T200 expression that occurs as B cell precursors differentiate.

The lymphoid subsets defined in our pediatric control population are quite similar to those reported in adults by others.[5,9] Control pediatric marrow contained predominantly mature T cells and a low number of B cells, a mixture of mature sIg$^+$ cells, and a few immature precursors. Specimens of resurgent marrow contained predominantly immature B cell precursors at multiple stages of differentiation and a lower percentage of T cells than controls. This pattern is very similar to that reported in fetal bone marrow.[9] Ryan et al[15] reported that the percentage and fluorescence intensity of CALLA$^+$ cells from children with immune thrombocytopenia, neutropenia, or nonhematologic malignancies not involving the marrow were no different than in children with ALL in remission. The present study shows clearly there is a difference in the percentage of B cell precursors between those with reactive processes and those on maintenance chemotherapy. The number of precursors in marrows from patients on chemotherapy depends on when the sample was obtained relative to receiving medication (data not shown). In the absence of more than one HLe-1 peak, the percentage of CALLA$^+$ cells was much lower. Thus it may be that the cases reported by Ryan's group[15] were actually reactive or resurgent. Similar to their findings, the present study shows that CALLA-bright cells are dim for HLe-1 and that CALLA-dim cells were brighter for HLe-1 but still dimmer than mature B cells.

Based on the comparative FI with each MoAb in resurgent marrows, a hypothetical scheme of B cell differentiation is proposed that incorporates not only the positivity with each MoAb but also the FI (Fig 6). The quantitative expression of each cell-surface antigen appears to be differentiation-related, as suggested by others.[10,15] The FI of HLA-DR is dimmer on immature cells and increases with maturation. The next antigen to appear is Leu-12. This antigen is dim on immature cells, increases in intensity during the pre-B stages, and loses some intensity on mature sIg$^+$ cells before disappearing completely on plasma cells. The CALLA (J5) antigen is first expressed after Leu-12 and is brightest on earlier precursors. A second population of more mature CALLA$^+$ cells quantitatively express less of the CALLA antigen before it disappears on mature B cells. Leu-16 first becomes positive on CALLA$^+$ cells and becomes brighter with further differentiation before it is lost at the plasma cell stage of differentiation.

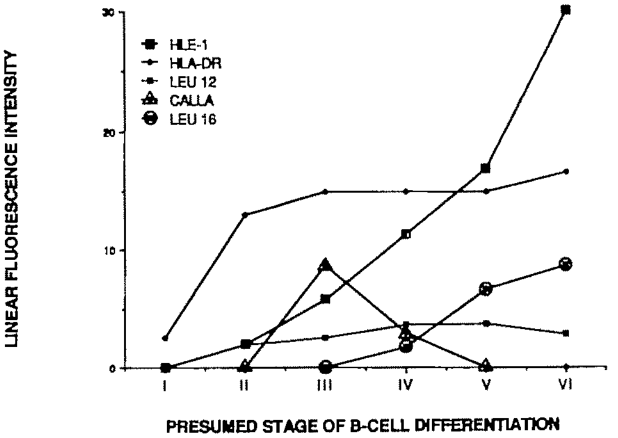

Fig 6. Proposed model of the relationships of quantitative surface antigen expression on maturing B cells. Fluorescence intensity is extrapolated from overall studies of resurgent marrow hyperplasia and peripheral blood. Stages I to VI of B cell differentiation represent a gradual transition from the least mature B cell precursor (I) to mature peripheral blood B cells (VI).

HLe-1 first appears on B cell precursors at about the same time as Leu-12. The FI is very low at this point but progressively increases to a maximum on mature peripheral blood B cells. Based on the present study it appears feasible to use HLe-1 FI as a scale of B cell differentiation.

Previous studies from this laboratory have documented quantitative alterations in expression of HLe-1 on malignant lymphoid cells.[1-3] The dimmest cells are those of ALL of non-T–non-B type, followed by B cell ALL, chronic lymphocytic leukemia (CLL), hairy cell leukemia (HCL), and normal peripheral blood B cells.[3] All cases of multiple myeloma have been negative for HLe-1 (data not shown). Thus quantitative expression of T200 correlates with the presumed level of cell-differentiation of these leukemic cells and normal mature B cells. Our studies also suggest that the quantitative expression of HLe-1 may relate to therapeutic outcome in some children with ALL.[2]

In summary, evaluation of the FI as a reflection of total surface-antigen content is a useful parameter for studies of cell differentiation. In particular, the quantity of T200 antigen provides information about the degree of B cell differentiation. If this antigen is quantitatively conserved on malignant B cells, it will provide a very useful marker of their differentiation state and provide a common framework on which to investigate relationships between normal and malignant B cells. Using this model it is feasible to evaluate malignant B cells to determine if they are really at a "frozen" stage of normal differentiation. The functional role of the T200 antigen on human B cells is not known, although the MoAbs have been reported to block cytolytic activity of natural killer cells[17,18] and cytotoxic T lymphocytes[22-25] and may inhibit B cell differentiation in mice.[23] Since the antigen increases in quantity during human B cell differentiation, it is tempting to speculate on its functional role during this process.

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Effect of Recombinant Interferons Alpha and Gamma on Human Bone Marrow-Derived Megakaryocytic Progenitor Cells

By Arnold Ganser, Carmelo Carlo-Stella, Jürgen Greher, Bernd Völkers, and Dieter Hoelzer

Interferons (IFNs) have been shown to suppress the proliferation of human pluripotent hematopoietic progenitor cells, CFU-GEMM, and committed erythroid (BFU-E, CFU-E) and granulocyte-macrophage (CFU-GM) progenitor cells. However, no information is yet available concerning the effect of IFNs on human megakaryocytic progenitor cells CFU-Mk. Furthermore the mechanisms underlying the inhibitory activity of IFNs are still controversial. Therefore highly purified recombinant IFN preparations, rIFN-alpha and rIFN-gamma, were assessed for their influence on in vitro growth of human bone marrow-derived CFU-Mk as well as CFU-GEMM. In addition, the role of hematopoietic accessory cells, that is, adherent cells and T lymphocytes, in the mediation of the suppressive effect of rIFNs was examined. When added to unseparated bone marrow cells, both rIFN preparations significantly inhibited colony formation with 50% inhibition of CFU-Mk occurring at 22 U/mL for rIFN-alpha and 59 U/mL for rIFN-gamma, while 50% inhibition of CFU-GEMM occurred at 59 U/mL for rIFN-alpha and 101 U/mL for rIFN-gamma. The suppressive effect of rIFN-alpha and rIFN-gamma was selectively abolished by monoclonal antibodies (MoAbs) against rIFN-alpha and rIFN-gamma, thus confirming that the inhibitory activity was due to the rIFN preparations used. The antiproliferative effect of rIFN-alpha and rIFN-gamma on CFU-GEMM growth was not associated with a decrease in the percentage of mixed colonies containing megakaryocytic cells as assessed by use of the MoAb C17.28 against platelet glycoprotein IIIa. Removal of adherent cells and T lymphocytes from the target bone marrow cells had no influence on the suppressive effect of rIFN-alpha, whereas it significantly reduced the inhibitory effect of rIFN-gamma on the growth of megakaryocytic colonies and the other hematopoietic progenitors. The data indicate that (1) human megakaryocytopoiesis is markedly inhibited by rIFN-alpha and rIFN-gamma, and (2) the inhibitory effect of rIFN-alpha is due to a direct action on hematopoietic progenitor cells, whereas the effect of rIFN-gamma is mediated to a significant degree through accessory cell populations.

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INTERFERONS (IFNs) are a group of naturally occurring, inducible proteins with antiviral,[1-3] antiproliferative,[4-6] immunomodulating,[7-10] and differentiative[11-15] effects. Furthermore they have been implicated in the suppression of normal hematopoiesis, as seen in aplastic anemia.[16,17] In clinical trials thrombocytopenia as well as granulocytopenia have been reported as the commonest hematologic side effects[18-20] in patients treated with IFNs.

IFNs have been shown to suppress the in vitro proliferation and/or differentiation of normal human pluripotent hematopoietic progenitor cells (CFU-GEMM, colony-forming-unit-granulocyte, erythrocyte, macrophage, megakaryocyte)[21,22] and of lineage-restricted progenitor cells (BFU-E, burst-forming-unit-erythroid; CFU-E, colony-forming-unit-erythroid; CFU-GM, colony-forming unit-granulocyte-macrophage).[5,22-30] While Dukes et al,[31] using a plasma clot-culture system, could show an inhibitory effect of natural IFN preparations on mouse megakaryocytic progenitor cells, no information is yet available concerning the effect of IFNs on human megakaryocytopoiesis in vitro. Furthermore the role of hematopoietic accessory cells, that is, monocytes-macrophages and T lymphocytes, in the mediation of the suppressive effects of IFNs is still controversial.[22,28]

It was therefore the aim of the present study to evaluate the in vitro effect of highly purified recombinant IFN-alpha (rIFN-alpha) and recombinant IFN-gamma (rIFN-gamma) on normal human megakaryopoiesis. A previously described in vitro clonal assay that promotes the growth of large, compact megakaryocytic colonies (CFU-Mk, colony-forming unit-megakaryocyte)[32-36] as well as CFU-GEMM, BFU-E, and CFU-GM was used. In addition, studies were performed to investigate whether the suppressive role of rIFNs on in vitro hematopoiesis was due to a direct effect on progenitor cells or whether it was mediated, at least in part, by hematopoietic accessory cells.

MATERIALS AND METHODS

Cell separation procedures. Bone marrow cells were obtained at the time of cardiac surgery from hematologically healthy donors who had given informed consent. Mononuclear light-density bone marrow cells (LDBMC) were separated by centrifugation on a Ficoll-Hypaque (FH) gradient (density 1.077 g/mL) at 400 g for 40 minutes at 20°C. Interface cells were washed three times in RPMI 1640 medium (GIBCO, Grand Island, NY) and suspended in Iscove's modified Dulbecco's medium (IMDM, Seromed, Berlin, FRG). Unless otherwise noted, LDBMC were cultured without further separation procedures.

In three separate experiments the effect of rIFNs was tested on unseparated LDBMC, nonadherent LDBMC (LDBMC-AC$^-$), T lymphocyte-depleted LDBMC (LDBMC-T$^-$), and nonadherent, T lymphocyte-depleted LDBMC (LDBMC-AC$^-$-T$^-$), derived from the same marrow samples. Removal of the adherent monocyte-macrophage cells (AC) was achieved by incubation in 25 cm^2 plastic tissue culture flasks (Nunc GmbH, Wiesbaden, FRG). Five milliliters of LDBMC (5×10^6/mL), suspended in RPMI 1640 medium with 20% fetal calf serum (FCS; Hyclone, Logan, UT), were placed

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in tissue culture flasks and allowed to adhere for two sequential one-hour periods at 37°C in a 5% CO_2 humidified atmosphere. Nonadherent cells (LDBMC-AC$^-$), collected by gentle agitation of the flask and careful aspiration of the supernatant, were washed twice in RMPI 1640 medium and suspended in IMDM before being assayed for hematopoietic cell growth.

Removal of T lymphocytes, either from LDBMC or from LDBMC-AC$^-$, was achieved by rosetting for two hours with 2-amino-ethylisothiouronium bromide-treated (AET; Sigma, Munich, FRG)[37] sheep red blood cells (SRBC; GMN, Walldorf, FRG)[38] at 4°C, followed by another density gradient centrifugation on FH (1.077 g/mL). T lymphocyte-depleted cell suspensions (LDBMC-T$^-$) were treated with 0.83% tris-buffered ammonium chloride, pH 7.2, washed three times in RPMI 1640 medium, and resuspended in IMDM before culture.

These cell separation procedures resulted in a nearly complete removal of adherent cells or T lymphocytes, as determined by immunologic analysis (see below) with monoclonal antibodies (MoAbs) MY4 (Coulter, Hialeah, FL) and OKT3 (Ortho Pharmaceutical, Raritan, NJ). Routinely, LDBMC-AC$^-$ were >97% MY4-negative and LDBMC-T$^-$ were >99% OKT3-negative. The viability of these cell fractions as determined by Trypan blue dye exclusion was always >95%.

Immunologic analysis of cell fractions and individual CFU-GEMM. The MoAbs OKT3, directed against mature T cells, (Ortho Pharmaceutical) and MY4, directed against monocytes-macrophages (Coulter, Hialeah, FL), were used to test the degree of purity of cell fractions obtained after adherent cell removal and T lymphocyte depletion.

The MoAb C17.28 against platelet glycoprotein IIIa,[39] kindly provided by Dr P. Lansdorp, Amsterdam, was used to identify megakaryocytic cells within individual mixed colonies that were picked up from nonconfluent areas of growth.

Immunologic characterization was performed according to Bross et al.[40] Briefly, the cells were transferred on to poly-L-lysin-coated (Sigma, Munich, FRG) spots on glass slides. After 30 minutes the attached cells were fixed with glutaraldehyde (0.05% in 0.1 mol/L phosphate buffer, pH 7.4) at 4°C for five minutes and washed with 0.3 mol/L glycine buffer for ten minutes. The cells were first incubated with the primary antibody (final dilution 1 40 in Hanks' balanced salt solution [HBSS] with 0.2% gelatine), and then goat antimouse IgG conjugated with alkaline phosphatase (Sigma, Munich, FRG), diluted 1·40 in HBSS/0.2% gelatine, was applied for 20 minutes The slides were developed for alkaline phosphatase with naphthol AS-MX-phosphate (Serva, Heidelberg, FRG) and Fast Red TR-salt (Serva, Heidelberg, FRG) and then mounted in glycerine gelatine. The percentage of positive cells was determined by counting at least 200 cells per reaction area.

In vitro assays. The assay for CFU-GEMM and CFU-Mk has been previously described.[32-36] Briefly, 1 × 10^5 LDBMC or cell fractions obtained after separation procedures were plated in 35-mm Petri dishes (Greiner, Nurtingen, FRG) in the presence and absence of different concentrations of rIFN-alpha and rIFN-gamma in 1-mL aliquots of IMDM containing 30% human fresh frozen plasma (FFP) from a single donor, 5% medium-conditioned by peripheral blood leukocytes (PHA-LCM)[41] in the presence of 1% phytohemagglutinin (PHA-HA 15, Burroughs Wellcome, Research Triangle Park, NC), 5 × 10^{-5} mol/L 2-mercaptoethanol (Sigma, Munich, FRG), 1.0 U erythropoietin (Step III, Connaught Laboratories, Toronto, Canada) and 0.9% (wt/vol) methylcellulose. After incubation for 14 days at 37°C in a humidified atmosphere supplemented with 5% CO_2, the cultures were examined with an inverted microscope. Four dishes were set up for each individual data point per experiment. Mixed colonies (CFU-GEMM) defined as containing at least erythroid and granulocytic cells by their in situ appearance, megakaryocytic colonies (CFU-Mk) defined as containing more than eight cells of varying size characterized by a translucent hyaline cytoplasm and highly refractile cytoplasmic border under the inverted microscope[34,35] Erythroid bursts (BFU-E) and granulocyte-macrophage colonies (CFU-GM) also grow in the culture plates; however, their growth was not the subject of this investigation. A single normal donor was used to provide human plasma throughout the whole study.

Experimental design. The effect of rIFN-alpha and rIFN-gamma on hematopoietic progenitor cell growth was evaluated as follows. (1) The different cell fractions obtained as described above were cultured throughout the entire incubation period with varying doses of rIFN-alpha and rIFN-gamma (10 to 10^4 U/mL). (2) LDBMC (4 × 10^6) were incubated with either control medium (IMDM, 10% fetal calf serum [FCS]) or medium (IMDM, 10% FCS) containing two different doses (10^3 and 10^4 U/mL) of rIFN-alpha and rIFN-gamma for 30 minutes at 37°C in 5% CO_2 humidified atmosphere. Then the cells were washed three times in RPMI 1640 medium, suspended in IMDM, and cultured for hematopoietic progenitor cell growth. (3) Neutralizing monoclonal antibodies (MoAbs) were added to culture dishes containing LDBMC and either rIFN-alpha (500 U/mL) or rIFN-gamma (500 U/mL) at various times after the initiation of culture

Recombinant IFNs and MoAbs against IFNs. rIFN-alpha (specific activity: 3 3 × 10^8 U/mg of protein) was kindly provided by Karl Thomae Co, Biberach, FRG; rIFN-gamma (specific activity: 2.4 × 10^7 U/mg of protein) was produced by Biogen Research Corp, Cambridge, MA and was kindly provided by Bioferon, Langheim, FRG. The interferon bioassay was performed in the HEP-2 cell line/Mengo virus assay.[42]

MoAbs EB-I against rIFN-alpha, GZ-4, and C-15 against rIFN-gamma, kindly provided by Karl Thomae Co, were used as a 1 : 200 dilution of mouse ascites. To neutralize the activity of rIFNs, MoAbs were incubated with rIFN preparations for 90 minutes at room temperature before culture

Statistical analysis. Four plates were scored for each data point per experiment, and the results were expressed as the mean ± 1 SEM. The Student's *t* test for paired data for the Scheffé multiple comparison test were used where appropriate to test the probability of significant differences between samples. IFN concentrations resulting in 50% inhibition of colony formation were calculated for each experiment by extrapolating from a least-square–linear-regression line relating the logarithm of the IFN concentration to the percentage of control colony formation. Statistical significance between the means of the logarithms of these concentrations were evaluated by the Student's *t* test.

RESULTS

Effect of rIFN-alpha and rIFN-gamma on LDBMC. When light-density bone marrow cells were exposed throughout the entire culture period to rIFN-alpha and rIFN-gamma, a statistically significant, dose-dependent suppression of colony growth from CFU-GEMM and CFU-Mk was seen (Fig 1) that was linear with the logarithm of the rIFN concentration over the tested range (10 to 10^4 U/mL). The degree of inhibition was essentially the same for both preparations of rIFN, with a 50% inhibition of CFU-Mk occurring at 22 U/mL for rIFN-alpha and 59 U/mL for rIFN-gamma, while 50% inhibition of CFU-GEMM occurred at 59 U/mL for rIFN-alpha and 101 U/mL for rIFN-gamma.

To determine whether or not the antiproliferative effect of rIFNs was associated with changes in the cellular composi-

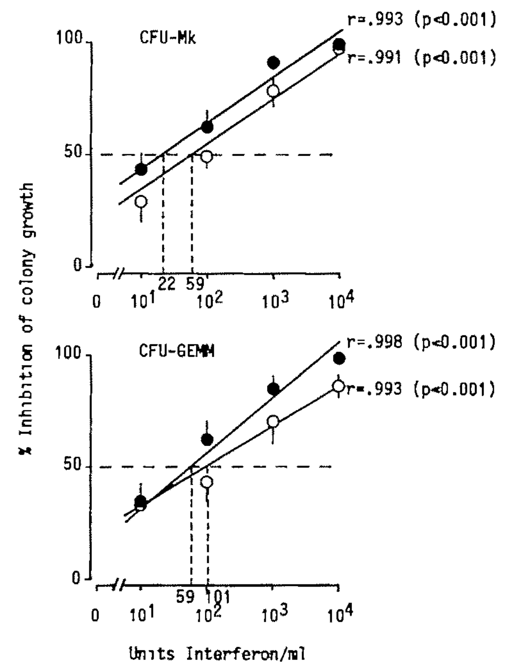

Fig 1. Effect of rIFN-alpha (●——●) and rIFN-gamma (○——○) on multipotent (CFU-GEMM) and megakaryocytic (CFU-Mk) progenitor cell growth in light-density bone marrow cells (LDBMC). Percentage of inhibition is expressed as the mean ± 1SEM from five (CFU-GEMM) and six (CFU-Mk) separate experiments, each involving different normal marrow donors. Control colonies per 1×10^5 LDBMC were 9 ± 1, 5 ± 0.6, 25 ± 1, 8.5 ± 2, and 5 ± 1 for CFU-GEMM; 5 ± 0.9, 20 ± 0.8, 3.0 ± 0.4, 21 ± 0.9, 25 ± 2, and 9 ± 0.6 for CFU-Mk. When compared to control cultures the inhibitory effect of rIFN-alpha was statistically significant at the dose of 10 U/mL on CFU-GEMM ($P < .05$) and CFU-Mk ($P < .01$); the inhibitory effect of rIFN-gamma was statistically significant at the dose of 10 U/mL on CFU-GEMM ($P < .001$) and CFU-Mk ($P < .05$) The linear regression lines for each rIFN were evaluated by the least-squares method, and the concentration of rIFN resulting in 50% inhibition of colony formation was extrapolated from these lines.

Table 1. Percentage of Bone Marrow CFU-GEMM Containing Megakaryocytic Elements*

Source	No. of Colonies Assayed		No. of Colonies Containing Megakaryocytic Elements†	
LDBMC		94		69 (73%; 57%-93%)‡
LDBMC +				
rIFN-alpha 10 U/mL	51	103	32	67 (65%; 50%-83%)‡
rIFN-alpha 10^2 U/mL	24		15	
rIFN-alpha 10^3 U/mL	19		14	
rIFN-alpha 10^4 U/mL	9		6	
LDBMC +				
rIFN-gamma 10 U/mL	46	153	24	95 (62%; 50%-76%)‡
rIFN-gamma 10^2 U/mL	59		42	
rIFN-gamma 10^3 U/mL	32		18	
rIFN-gamma 10^4 U/mL	16		11	

*All CFU-GEMM contained erythroid cells by in situ appearance

†Analyzed immunologically with the C17 28 MoAb against platelet glycoprotein IIIa

‡95% Confidence interval.

tion of mixed colonies, the percentage of CFU-GEMM containing megakaryocytic elements was assessed using the MoAb C17.28 against platelet glycoprotein IIIa.[39] From Table 1 it can be seen that the percentage of CFU-GEMM containing megakaryocytic elements was unaffected by either rIFN-alpha or rIFN-gamma. The results were comparable for all different dose ranges of rIFNs. Similarly, the cellular composition of the CFU-GEMM was unaffected by the depletion of T cells and/or adherent cells.

Three MoAbs were assessed for their capacity to neutralize the effects of rIFNs on colony growth. The data given in Table 2 show that the MoAb against rIFN-alpha inactivated the suppressive action of rIFN-alpha but failed to eliminate the suppressive effect of rIFN-gamma. On the other hand, and two MoAbs against rIFN-gamma inactivated the action of rIFN-gamma but not that of rIFN-alpha.

The effect of short-term exposure to rIFNs was studied by incubating LDBMC with different doses of rIFN-alpha and rIFN-gamma for 30 minutes (Table 3). Such a short exposure failed to cause a significant reduction in colony growth, and even when 10^4 U/mL of rIFNs were used, the inhibition of hematopoietic progenitor cells was <35%.

To further investigate the mode of action of rIFNs on hematopoietic progenitor cells, neutralizing antibodies were added to the culture dishes at various time intervals after the start of the in vitro cultures (Table 4). The inhibition exerted both by rIFN-alpha and rIFN-gamma could be abrogated by the addition of neutralizing MoAbs as late as one hour after the onset of incubation but was no longer reversible after four hours. The only exception was the inhibitory effect of rIFN-alpha on CFU-Mk, which was still fully reversible at 4 hours by the addition of the neutralizing antibody.

Effect of rIFN-alpha and rIFN-gamma on LDBMC-AC⁻, LDBMC-T⁻, and LDBMC-AC⁻-T⁻. Since various cell types, collectively termed hematopoietic accessory cells,

Table 2. Selective Inactivation of Suppressive Effects of rIFN-Alpha and rIFN-Gamma on Hematopoietic Progenitor Cell Growth (Mean ± SEM) by Preincubation of rIFNs With Their Respective MoAbs*

	CFU-GEMM	CFU-Mk
Control medium	8 5 ± 0.3	9.8 ± 0 5
rIFN-alpha (10^3 U/mL)	*0.5 ± 0.3*†	*0.7 ± 0.2*§
rIFN-gamma (10^3 U/mL)	*2.2 ± 0 3*†	*3 2 ± 0.5*§
Anti-rIFN-alpha (EB-1)	8 5 ± 0 5	8 8 ± 1.2
Anti-rIFN-gamma (GZ-4)	8.5 ± 0 9	8.7 ± 0 5
Anti-rIFN-gamma (C-15)	8.5 ± 0 5	9.2 ± 1 1
Anti-rIFN-alpha (EB-1) + rIFN-alpha	*7.3 ± 0.2*‖	*12 ± 0.6*‖
Anti-rIFN-gamma (GZ-4) + rIFN-gamma	*8.2 ± 0 2*‖	*7.5 ± 0.6*‖
Anti-rIFN-gamma (C-15) + rIFN-gamma	*9.2 ± 0.7*‖	*10 ± 0 6*‖
Anti-rIFN-alpha (EB-1) + rIFN-gamma	2.5 ± 0.5§	4.5 ± 0 9‡
Anti-rIFN-gamma (GZ-4) + rIFN-alpha	0.2 ± 0.2†	1 0 ± 0 4†

*Colony growth per 1×10^5/mL light-density–mononuclear bone marrow cells in the absence and presence of 10^3 U/mL of rIFN (preincubated for 90 minutes at room temperature with and without 10^3 neutralizing units of MoAb against rIFN-alpha or rIFN-gamma).

†$P <$ 001

‡$P < .02$.

§$P < .01$

‖P – not significant.

Table 3. Effect of Preincubation With rIFN-Alpha and rIFN-Gamma on CFU-GEMM and CFU-Mk Progenitor Cell Growth*

	% Inhibition†	
IFN	CFU-GEMM	CFU-Mk
rIFN-alpha		
10^3 U/mL	27 ± 13	9 ± 14
10^4 U/mL	21 ± 21	34 ± 13
rIFN-gamma		
10^3 U/mL	0 ± 19	0 ± 10
10^4 U/mL	20 ± 24	17 ± 23

*Cells were incubated with the indicated doses of rIFN or medium alone for 30 minutes (37°C, 5% CO_2), washed, and cultured in methylcellulose (see text for details).

†Percentage of inhibition is expressed as the mean ± 1 SEM from four separate experiments, each involving a different normal marrow donor. Control colonies per 1 × 10^5 LDBMC were 4 5 ± 0.6, 13.5 ± 1, 9 ± 2, and 13.5 ± 1 for CFU-GEMM, 4.5 ± 0.3, 9.5 ± 1, 19 5 ± 0.5, and 21 ± 3 for CFU-Mk. The differences were not significant, as evaluated by the Scheffé multiple comparison test.

are involved in the regulation of hematopoietic progenitor cell growth,[43] the influence of rIFN on LDBMC depleted of accessory cells was studied.

The effect of rIFN-alpha on LDBMC-AC^-, LDBMC-T^-, and LDBMC-AC^--T^- is summarized in Table 5. A dose-dependent inhibition of growth of both CFU-Mk and CFU-GEMM was seen with rIFN-alpha, with a 50% inhibition concentration comparable to that observed when rIFN-alpha was added to LDBMC cultures, and no statistically significant differences were detected. On the other hand, the inhibitory effect of rIFN-gamma was significantly reduced after depletion of adherent cells and/or T cells with a 50% inhibitory concentration falling outside the upper limit of 10^4 U/mL studied in our experiments (Table 6). The effect of depletion of T cells appeared to more completely remove the ability of rIFN-gamma to inhibit either type of colony studied (Table 5).

DISCUSSION

The experiments presented here are, to our knowledge, the first that describe the suppressive effect of human rIFN-alpha and rIFN-gamma on human megakaryocytic progenitor cells and on the even more immature pluripotent CFU-GEMM, comparing the effect on both unseparated and accessory cell-depleted bone marrow cells. While no data at all exist on the effect of IFNs on CFU-Mk, previous studies of its effect on CFU-GEMM were done with IFNs purified from cell culture supernatants[21,22] instead of cloned IFNs, as in our experiments. Furthermore fresh frozen human plasma was substituted for FCS in our assay system, allowing not only the in vitro growth of megakaryocytic colonies[33-35] but also supporting megakaryocytic maturation within mixed colonies, as detected by immunologic analysis of single colonies.[36]

Both rIFN-alpha and rIFN-gamma are potent inhibitors of human megakaryopoiesis suppressing the in vitro growth of the progenitors of megakaryocytes, CFU-GEMM and CFU-Mk. The dose-dependence of this suppression is similar to that observed for erythroid and granulocyte-macrophage progenitor cells as found in our own experiments (data not shown) and by other authors previously.[5,22-30]

Although the hematopoietic progenitor cells have routinely been exposed to rIFNs throughout the entire culture period, our experiments demonstrate that the inhibitory effect is not observed if the rIFNs are either washed out or neutralized by MoAbs within the first hour of exposure. While the inhibition could partially be abrogated by adding neutralizing antibodies four hours after the initiation of the cultures, no further neutralization could be obtained after 24 hours. This is in agreement with the observation that IFNs exert their action by binding to membrane receptors and that

Table 4. Inactivation of Suppressive Effects of rIFN-Alpha and rIFN-Gamma on Hematopoietic Progenitor Cell Growth (Mean ± 1 SEM) by Addition of Neutralizing MoAbs*

	Time of Addition of Neutralizing Antibody	CFU-GEMM	CFU-Mk
Control medium		20 ± 3	29 ± 4
+ rIFN-alpha (500 U/mL)		4.5 ± 0.5‡	4 ± 1‡
+ rIFN-alpha + EB-1	−1 5 h	16 ± 2	27 ± 9
+ rIFN-alpha + EB-1	1 h	9.5 ± 3.5†	25 ± 4
+ rIFN-alpha + EB-1	4 h	8 ± 1‡	20 ± 5
+ rIFN-alpha + EB-1	24 h	3 ± 0 3‡	6 ± 0.3‡
+ rIFN-alpha + EB-1	96 h	0‡	4.5 ± 0.5‡
+ rIFN-gamma (500 U/mL)		5 ± 1‡	6 ± 2‡
+ rIFN-gamma + GZ-4	−1 5 h	16 ± 2	20 ± 1
+ rIFN-gamma + GZ-4	1 h	16 ± 3	26 ± 4
+ rIFN-gamma + GZ-4	4 h	11.5 ± 1.5‡	7 ± 1‡
+ rIFN-gamma + GZ-4	24 h	4.5 ± 0 5‡	8 ± 2‡
+ rIFN-gamma + GZ-4	96 h	4.5 ± 0.5‡	4 5 ± 0.5‡

*Colony growth per 1 × 10^5/mL light-density bone marrow cells. Five hundred neutralizing units of monoclonal antibodies against rIFN-alpha (EB-1) or rIFN-gamma (GZ-4) were added in a volume of 100 uL to the 1-mL dishes at various times after the onset of the incubation period.

†$P < .05$.

‡$P < .01$.

Table 5. Influence of Removing T Lymphocytes and Monocytes From Normal Human Bone Marrow on Inhibition of CFU-GEMM and CFU-Mk Progenitor Cell Growth by rIFN*

	% Inhibition							
	CFU-GEMM				CFU-Mk			
rIFN (U/mL)	LDBMC	LDBMC-AC$^-$	LDBMC-T$^-$	LDBMC-AC$^-$T$^-$	LDBMC	LDBMC-AC$^-$	LDBMC-T$^-$	LDBMC-AC-T$^-$
rIFN-alpha								
10^1	26 ± 13,ns	28 ± 6†	31 ± 6†	21 ± 9,ns	50 ± 14,ns	28 ± 3†	36 ± 4	36 ± 7†
10^2	61 ± 4†	47 ± 7†	50 ± 8†	43 ± 8†	64 ± 4†	46 ± 5†	65 ± 4†	62 ± 6†
10^3	79 ± 9†	65 ± 5†	79 ± 5†	75 ± 8†	90 ± 3†	71 ± 9†	84 ± 5†	84 ± 6†
10^4	98 ± 2†	95 ± 3†	99 ± 1†	97 ± 3†	99 ± 1†	92 ± 5†	99 ± 1†	98 ± 2†
rIFN-gamma								
10^1	31 ± 2†	18 ± 5,ns	6 ± 6,ns	10 ± 5,ns	31 ± 15,ns	18 ± 9,ns	2 ± 2,ns	15 ± 8,ns
10^2	30 ± 5†	17 ± 1†	6 ± 6,ns	3 ± 3,ns	52 ± 7†	18 ± 12,ns	1 ± 1,ns	24 ± 12,ns
10^3	58 ± 10†	17 ± 4,ns	6 ± 6,ns	9 ± 3,ns	69 ± 8†	29 ± 3†	14 ± 14,ns	20 ± 12,ns
10^4	82 ± 4†	53 ± 4	34 ± 17,ns	32 ± 2†	95 ± 1†	55 ± 8†	23 ± 17,ns	35 ± 12,ns

Abbreviations ns, no significant change, $P > .05$

*The results are expressed as % inhibition ± 1 SEM compared with control medium (−IMDM) from three separate sets of experiments and performed concomitantly on unseparated light-density bone marrow cells (LDBMC), nonadherent LDBMC (LDBMC-AC$^-$), T lymphocyte-depleted LDBMC (LDBMC-T$^-$), and nonadherent–T lymphocyte-depleted LDBMC (LDBMC-AC$^-$T$^-$) derived from the same marrow sample. Control numbers of CFU-GEMM ranged from 8.5 to 25 for LDBMC, 3 to 6 5 for LDBMC-AC$^-$, 5.8 to 17 for LDBMC-T$^-$, and 4 5 to 8 for LDBMC-AC$^-$-T$^-$ Control numbers of CFU-Mk ranged from 9 to 25 for LDBMC, 7 to 17 for LDBMC-AC$^-$, 14 to 21 for LDBMC-T$^-$, and 8 to 24 for LDBMC-AC$^-$-T$^-$.

†Percent significant change from control, *P* value at least <.05 (two-tailed *t* test).

they are rapidly internalized and degraded at 37°C.[44] Although we do not know at which stage in the maturation pathway IFNs exert their suppressive effect, experiments with BFU-E and the even more mature CFU-E would suggest a loss of susceptibility to rIFN-gamma with progenitor cell maturation.[45]

However, immunologic analysis of single mixed colonies with the MoAb C17.28 against platelet glycoprotein IIIa[39] did not reveal any inhibition by rIFN-alpha or rIFN-gamma in the in vitro maturation of the pluripotent progenitors along the megakaryocytic lineage. Whether or not the bone marrow cells were cultured in the presence on IFNs, between 62% and 73% of the mixed colonies contained megakaryocytic elements. These findings indicate that IFNs mainly exert an antiproliferative action without influencing megakaryocytic differentiation at the levels analyzed in the experiments.

Table 6. Influence of Removing T Lymphocytes and Monocytes From Normal Human Bone Marrow on the 50% Inhibition Values*

		CFU-GEMM	CFU-Mk
IFN	Cell Fraction	50% Inhibition at (U/mL)	
rIFN-alpha	LDBMC	45 $\overset{\times}{\div}$ 1.6	19 $\overset{\times}{\div}$ 1 5
	LDBC-AC$^-$	92 $\overset{\times}{\div}$ 1.2	106 $\overset{\times}{\div}$ 1.6
	LDBMC-T$^-$	52 $\overset{\times}{\div}$ 1 2	30 $\overset{\times}{\div}$ 1.6
	LDBMC-AC$^-$-T$^-$	89 $\overset{\times}{\div}$ 1.7	26 $\overset{\times}{\div}$ 1.7
rIFN-gamma	LDBMC	298 $\overset{\times}{\div}$ 1 7	93 $\overset{\times}{\div}$ 1.8
	LDBC-AC$^-$	>10^4†	>10^4†
	LDBMC-T$^-$	>10^4†	>10^4†
	LDBMC-AC$^-$-T$^-$	>10^4†	>10^4†

*The results are expressed as the geometric mean $\overset{\times}{\div}$ 1 SEM of the rIFN concentrations resulting in 50% inhibition of colony formation in three separate experiments (see Table 5).

†Significantly different from control (LDBMC); *P* value at least <.05 (two-tailed *t* test for paired data)

Our data strongly indicate that, similar to the findings on BFU-E,[28] the mechanisms underlying the suppressive effect of the two types of rIFNs on CFU-GEMM and CFU-Mk are different. Recombinant IFN-alpha apparently acts directly on the progenitor cells, although regeneration of the depleted T lymphocytes and macrophages cannot be ruled out, whereas the effect of rIFN-gamma is largely mediated by accessory cells. These are usually T lymphocytes but may also be adherent cells, as shown by the lower degree of growth inhibition when rIFN-gamma was added to LDBMC-AC$^-$.

Two lines of evidence suggest that the observed suppressive effects were actually caused by IFN-alpha and IFN-gamma. First, recombinant DNA-derived IFNs were used that were highly purified, in contrast to previous studies using partially purified natural IFN preparations[5,21-27] in which contamination with a variety of biologically active molecules could not be ruled out.[46] Second, the antiproliferative effect of both rIFN preparations was selectively abolished by the respective MoAbs against rIFN-alpha and rIFN-gamma, thus confirming that the inhibitory effect was due to the rIFN used.

The concentrations of rIFNs that inhibit the in vitro colony formation of CFU-GEMM and CFU-Mk in our assay system are in the same range as previously described for the erythroid progenitors BFU-E and the granulocyte-macrophage CFU-GM.[5,21-30] Whether suppression of hematopoiesis by IFNs also occurs in vivo under physiologic conditions remains to be determined.

The role of accessory cells, as found in our experiments, is in agreement with recently reported observations[47-48] demonstrating that IFN-gamma can activate macrophages, lymphocytes, and natural killer cells to suppress hematopoietic colony formation in vitro.

However, at lower concentrations rIFN-gamma may act as a positive stimulus in vivo by enhancing the release of colony-stimulating factors from lymphocytes[49] and monocytes.[50,51] The effect of in vivo certainly is a result of many complex cell–cell or molecule–cell interactions, and the positive effect of rIFN-gamma at low concentrations could eventually be counterbalanced by the induction of responsiveness of cells to inhibitory molecules like tumor necrosis factor[52] or the activation of inhibitory cells.

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Chloramphenicol-Induced Bone Marrow Injury: Possible Role of Bacterial Metabolites of Chloramphenicol

By Joaquin J. Jimenez, Grace K. Arimura, Wafa H. Abou-Khalil, Mike Isildar, and Adel A. Yunis

To explore the potential role of some bacterial metabolites of chloramphenicol (CAP) in CAP-induced hematotoxicity, we examined their cytotoxic effects on bone marrow cells in vitro using a number of cytotoxicity parameters. Among the metabolites tested, dehydro-CAP (DHCAP) and p-nitrophenyl-2-amino-3 hydroxypropanone-HCl (NPAP) were more toxic than CAP. DHCAP was at least as toxic as nitroso-CAP. At concentrations of $\leq 10^{-4}$ mol/L, DHCAP caused total irreversible inhibition of myeloid colony (CFU-GM) growth and 80% inhibition of DNA synthesis in human bone marrow. Incubation of human bone marrow cells with 10^{-4} mol/L nitroso-CAP or DHCAP for 24 hours resulted in 75% and 65% cell death respectively. Although DHCAP was 10- to 20-fold more cytotoxic than CAP, it was only one third as effective in inhibiting mitochondrial protein synthesis, indicating that DHCAP exerts its toxic effect by alternate mechanisms. The cytotoxicity of DHCAP and its relative stability, compared to the unstable nitroso CAP, suggest that this bacterial metabolite of CAP, and possibly others, may play a significant role in CAP-induced hematotoxicity.

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EVIDENCE has recently been presented suggesting that nitroreduction of chloramphenicol (CAP) may play an important role in hematologic toxicity from the antibiotic. Thus nitroso-CAP, a nitroreduction intermediate of CAP, was found to be much more cytotoxic in vitro than CAP.[1-5] In micromolar concentrations, nitroso-CAP suppresses CFU-GM growth irreversibly, inhibits DNA synthesis in bone marrow, and causes damage to isolated DNA in vitro and to DNA in intact cells.

Although nitroreduction of CAP can be carried out by the liver,[6-8] presumably resulting in the production of the toxic nitroso-intermediate, it appears unlikely that nitroso-CAP can reach its target organ, the bone marrow, because of extreme instability.[8,9] Thus nitroso-CAP is eliminated from human whole blood within seconds,[9] and no nitroso-CAP passed through rat liver in intact form.[8]

In man metabolic transformation of CAP may take place in liver[6,7] or by intestinal bacteria.[10] A number of metabolites of CAP have been identified that are produced by bacteria known to reside in the intestinal tract. The potential role of these metabolites in hematotoxicity from CAP has not been assessed. In the present study we compared the effects of some of these metabolites on a number of parameters in vitro, including cell viability, CFU-GM growth, DNA synthesis, and mitrochondrial protein synthesis. Two metabolites were found to be much more toxic than CAP: dehydro-CAP (DHCAP) and p-nitrophenyl-2-amino-3-hydroxypropanone HCl (NPAP). The possible significance of these observations in relation to reversible hematotoxicity and aplastic anemia from CAP are discussed.

MATERIALS AND METHODS

CAP, nitroso-CAP, NPAP, and DHCAP were kindly provided by Dr D. Della Bella of Zambon, S.p.A., Milan, Italy. Tritiated thymidine and ^{14}C-leucine were obtained from New England Nuclear, Boston. Dulbecco's modified Eagles medium (DMEM) culture media was purchased from Flow Biological, McLean, VA; horse serum and fetal calf serum (FCS) were purchased from the University of Miami Comprehensive Cancer Center; Diethylamino ethyl-dextran and Histopaque 1077 were obtained from Sigma Chemical Co, St Louis; and Bacto-Agar from Difco Laboratories, Detroit. C57 BL mice were supplied by Jackson Laboratories, Bar Harbor, ME. Human bone marrow was obtained from healthy volunteers by aspiration from the posterior iliac crest after informed consents had been obtained according to the principles of the Declaration of Helsinki. All materials used to measure ^{14}C-leucine incorporation into mitochondrial protein were sterilized either by autoclaving or by filtration through Millipore filters with a pore size of 0 45 µm.

Human marrow CFU-GM assay The assay for CFU-GM was carried out according to the method of Bradley and Metcalf,[11] as described previously. All assays were done in triplicate. Briefly, 2 × 10^5 nucleated human bone marrow cells were plated in 35 × 10-mm Falcon petri dishes (Falcon Plastics, Oxnard, CA) in 0.3% Bacto-Agar with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 10% horse serum. Drugs and colony-stimulating factor were added directly to the plates prior to plating. GCT (GIBCO, Grand Island NY)-conditioned medium was used as a source of colony-stimulating factor. To test for reversibility, 1 × 10^6 marrow cells were incubated in medium with and without drug for the specified period, followed by washing and assay with and without drug. To test for viability, 5 × 10^5 marrow cells were incubated in medium with and without drug for the specified period. Cell viability was determined by the trypan blue exclusion test.

Mouse marrow assay. C57 BL/2J mice were killed by cervical dislocation, and the femur was removed under sterile conditions. The marrow was flushed from the femur shaft with 2 mL of DME supplemented with 10% FCS. Double-strength DME supplemented with 20% FCS and 20% horse serum was added 1 : 1 to a 0.6% liquid agar solution. Cells were added to a final concentration of 1 × 10^5 cells/mL. This mixture was suplemented with diethyl aminoethyl (DEAE)-dextran (0.075 mg/mL) and L-asparagine (0.02 mg/mL). Drugs and colony-stimulating factor were added directly to the plates prior to plating. Mouse lung-conditioned medium was used as a source of CSF.[12]

DNA synthesis. DNA synthesis was assayed in human bone marrow cells by measuring the incorporation of tritiated thymidine into trichloroacetic acid (TCA)-precipitable material as previously described.[13] The buffy coat from human bone marrow was sus-

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pended in HBSS to give a cell concentration of 5 to 10 × 10^6 cells/mL. Incubations were carried out in 1-mL-volumes including 0.8 mL of cell suspension plus additions. Drugs dissolved in dimethyl sulfoxide (DMSO) were added to the designated concentration in a volume not exceeding 0.1 mL. All incubations were carried out in duplicates. After a preincubation period of 30 minutes, 1 µCi of ^{3}H-thymidine was added, and incubation was continued for one hour. The cells were washed once with 5 mL of saline, and the red cells were hemolyzed by one wash in 5 mL of 3% glacial acetic acid. The cell button was then washed successively at 5°C in 5 mL-volumes of 10% TCA, 95% ethyl alcohol, and ethyl ether. The pellets were dried in air and dissolved in 1 mL of NaOH followed by the addition of 10 mL of scintillation fluor for counting. Radioactivity was expressed as counts per minute per sample.

Isolation of mitochondria from bone marrow. Adult New Zealand white rabbits weighing 2 to 3 kg were used Rabbits were killed by decapitation, and the two long bones from each hind leg and humerus from each foreleg were excised and stripped of all muscle; the marrow was removed, placed in cold homogenizing medium, and weighed. All subsequent steps were carried out in a cold room at 4°C.

Bone marrow mitochondria were isolated according to Weinbach[14] with slight modification. The marrow was homogenized (1 g of marrow/8 mL) in a medium consisting of 0.25 mol/L sucrose, 2 mmol/L EDTA, 2 mmol/L nicotinamide, and 1 mg/mL of bovine serum albumin (BSA), adjusted to pH 7.4 with KOH. The homogenization was accomplished in a Potter-Elvehjem glass homogenizer with the Teflon pestle mounted on a three-eighth inch variable speed electric drill at a low setting (about 600 rpm) until an apparently homogeneous suspension was obtained (4 to 5 plunges). The homogenate was centrifuged at 800 g for ten minutes, and the supernatant with a thick layer of fat on the top was filtered through two double layers of sterile gauze and recentrifuged as before. The resulting supernantant was centrifuged at 8,500 g for ten minutes to collect the mitochondria. The mitochondrial pellet was washed twice by suspending it in 2 mL homogenizing medium/g of marrow and centrifuging at 8,500 g for ten minutes. The final pellet containing the mitochondria was resuspended in the homogenizing medium to give a final concentration of 40 mg protein/mL.

Assay of mitochondrial protein synthesis. The assay of protein synthesis was carried out at 30°C in the following incubation medium: 50 mmol/L bicine, 15 mmol/L KPi, 10 mmol/L succinate, 1 mmol/L EDTA, 50 mmol/L KCl, 5 mmol/L ATP, 5 mmol/L nicotinamide, 6 mmol/L $MgCl_2$, 50 units penicillin G/mL adjusted to pH 7.4 with KOH, 60 µg of a complete amino acid mixture minus leucine, as described by Roodyn et al,[15] and mitochondria 0.75 mg protein in a total volume of 0 225 mL. Incubation was carried out in a Dubnoff metabolic shaker After two minutes of preincubation, 2 µCi L-^{14}C-leucine (specific activity 339 mCi/mmol) was added. At appropriate time intervals aliquots of 0.1 mL were transferred to Whatman No. 3 paper discs and were washed and counted according to the method of Mans and Novelli.[16] Inhibition of the incorporation of ^{14}C-leucine in the presence of 300 µg/mL of cycloheximide (10% to 20%) due to microsomal contamination constituted a background and was subtracted from all assays.

RESULTS

Effect of CAP and analogues on the growth of mouse marrow CFU-GM. The results of three experiments testing the effect of various concentrations of CAP, nitroso-CAP, NPAP, and DHCAP (see Fig 1 for structures) on murine CFU-GM growth are presented in Table 1. All three analogues of CAP were much more inhibitory than CAP. At concentrations of 5 × 10^{-5} mol/L DHCAP and nitroso-CAP caused complete inhibition of growth compared to a very slight inhibition by CAP. Among the three analogues, DHCAP was the most inhibitory; even at the low concentration of 10^{-6} mol/L DHCAP caused almost complete inhibition. NPAP was the least inhibitory, and nitroso-CAP was intermediate.

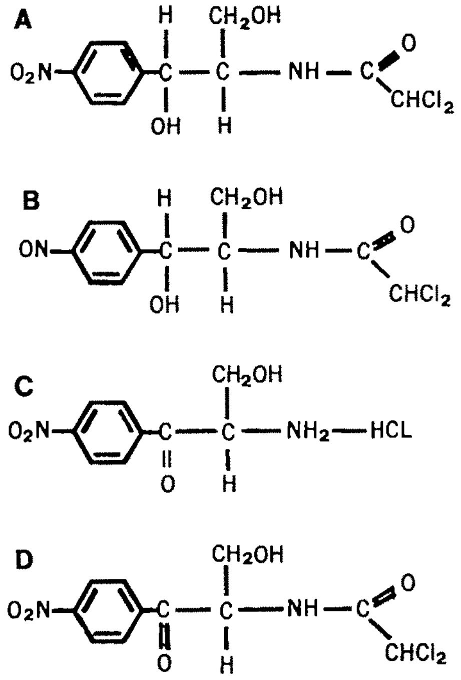

Fig 1. Chloramphenicol and its analogues. (A) CAP, 1-p-nitrophenyl-2-dichlodichloroacetamido-1,3-propanediol; (B) Nitroso-CAP, 1-p-nitrosophenyl-2-dichloroacetamido-1,3-propanediol; (C) NPAP, p-nitrophenyl-2-amino-3-hydroxypropanone HCl; (D) DHCAP, 2-dichloroacetamido-3-hydroxypropio-p-nitrophenone.

Effect of CAP and analogues on the growth of human CFU-GM. The results of four separate experiments in human bone marrow, shown in Table 2, were qualitatively similar to those in mouse marrow. Among the three CAP analogues, DHCAP was the most toxic, causing significant inhibition of growth (25%) at the low concentration of 10^{-7} mol/L and reaching 100% inhibition at 5 × 10^{-6} mol/L. At this latter concentration nitroso-CAP caused only about 40% inhibition. NPAP was less inhibitory; at 10^{-5} mol/L there was 69% inhibition compared to almost complete inhibition by nitroso-CAP. A composite graph showing the relative effect of various concentrations of CAP and analogues on the growth of human CFU-GM is shown in Fig 2. All three analogues were over 20-fold more inhibitory than CAP.

Reversibility of inhibition. Figure 3 shows the degree of reversibility of inhibition of human CFU-GM growth upon removal of drug. As has been shown previously with nitroso-CAP,[2] inhibition by DHCAP was largely irreversible. When lower inhibitory concentrations were used, that is, 5 × 10^{-6} mol/L nitroso-CAP or 5 × 10^{-7} M DHCAP, slightly better reversibility was observed. Significantly greater reversibility was observed with NPAP. Inhibition by CAP was completely reversible, as also has been demonstrated previously.[2]

Table 1. Effect of CAP and Analogues on In Vitro Growth of Mouse CFU-GM

Drug (M)		Colonies/10^5 Nucleated Cells (± SEM) Experiment 1	Experiment 2	Experiment 3	Experiment 4
	0	103 ± 2	114 ± 6	92 ± 2	91 ± 3
CAP	5×10^{-5}	95 ± 1	—	70 ± 2	—
	10^{-4}	—	—	—	55 ± 3
Nitroso-CAP	10^{-5}	—	—	—	13 ± 2
	2×10^{-5}	—	10 ± 3	—	2
	5×10^{-5}	0	—	0	0
NPAP	10^{-5}	—	56 ± 3	—	43 ± 2
	2×10^{-5}	—	13 ± 1	—	14 ± 1
	5×10^{-5}	0	—	6 ± 1	0
DHCAP	10^{-6}	—	—	—	8 ± 2
	5×10^{-6}	—	—	—	0
	10^{-5}	—	0	—	0
	5×10^{-5}	0	—	0	—

Table 2. Effect of CAP and Analogues on Human Marrow CFU-GM

Drug (M)		Colonies/2×10^5 Nucleated Cells (± SEM) Experiment 1	Experiment 2	Experiment 3	Experiment 4	Mean % Inhibition
	0	76 ± 2	89 ± 2	77 ± 3	80 ± 2 5	0%
CAP	5×10^{-5}	69 ± 2	—	—	—	15%
	10^{-4}	55 ± 2	62 ± 3	59 ± 2	—	28%
	2×10^{-4}	26 ± 3	33 ± 2	36 ± 3	37 ± 4	61%
	3×10^{-4}	12 ± 3	—	17 ± 2	—	82%
Nitroso-CAP	5×10^{-6}	26 ± 3	—	24 ± 3	—	39%
	10^{-5}	1 ± 1	4 ± 2	2 ± 1	4 ± 1.5	96%
	2×10^{-5}	0	0	0	—	100%
	5×10^{-5}	—	0	—	0	100%
NPAP	10^{-5}	14 ± 1	—	23 ± 2	22 ± 2	75%
	2×10^{-5}	5 ± 1	11 ± 2	12 ± 2	—	89%
	5×10^{-5}	0	0	—	0	100%
DHCAP	10^{-7}	—	—	—	60 ± 2 5	25%
	5×10^{-7}	—	—	24 ± 2	21 ± 2	72%
	10^{-6}	21 ± 5	—	11 ± 2	15 ± 3.5	80%
	5×10^{-6}	0	0	0	0	100%
	10^{-5}	0	0	0	0	100%

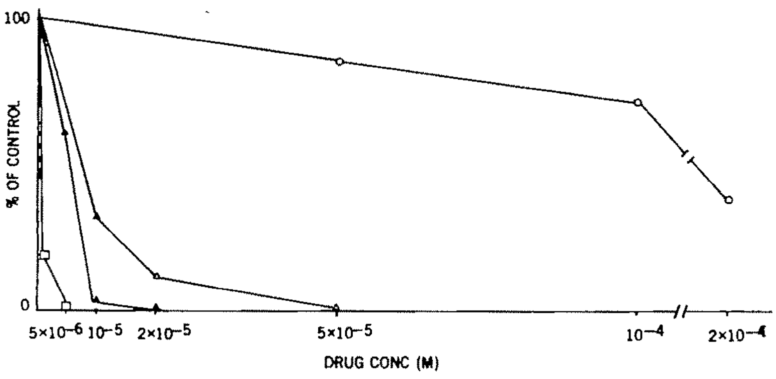

Fig 2 Comparative effect of CAP and analogues on the growth of human marrow CFU-GM in vitro. Each plot represents the composite of three separate experiments. CAP, ○; NPAP, △; DHCAP, □; Nitroso-CAP, ▲.

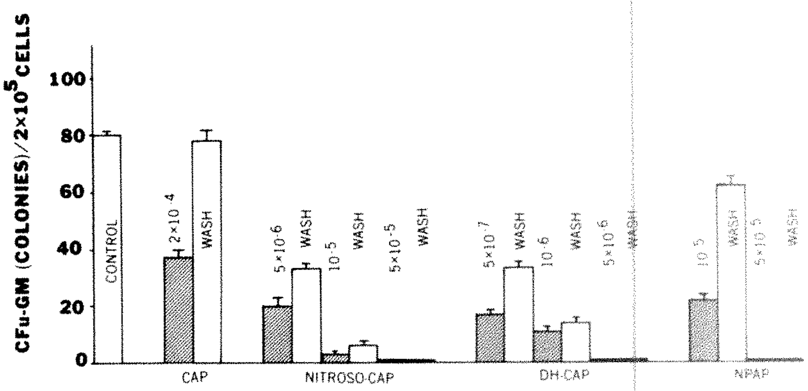

Fig 3. Reversibility of drug effect on human CFU-GM growth. Marrow cells were preincubated with drug (at the indicated concentrations) and without for eight hours, followed by washing, then cultured at 2×10^5 viable cells/per assay.

Effect on bone marrow cell viability. Figure 4 shows the effect of CAP and analogues on the viability of human bone marrow cells after 12 and 24 hours of incubation. Both nitroso-CAP and DHCAP caused over 60% cell death after 24 hours. NPAP was much less effective, while CAP, as has been shown before, had no effect on cell viability.

Effect of CAP and analogues on DNA synthesis in human marrow. CAP has been shown previously to inhibit DNA synthesis only when concentrations approaching 10^{-3} mol/L are used. Figure 5 shows the results of an experiment comparing the effect of 10^{-4} mol/L nitroso-CAP, DHCAP, and NPAP with that of 2×10^{-3} mol/L CAP. All three analogues inhibited DNA synthesis at 10^{-4} mol/L; DHCAP was the most inhibitory, followed by nitroso-CAP; NPAP was less effective.

Effect of CAP analogues on mitochondrial protein synthesis. The effect of CAP, nitroso-CAP, DHCAP, and NPAP, each at 10 μg/mL, is shown in Table 3. As is known from previous studies, CAP is a potent inhibitor of mitochondrial protein synthesis, causing 80% inhibition at 10 μg/mL. All three analogues were considerably less inhibitory than CAP; DHCAP was the least potent, causing only 25% inhibition.

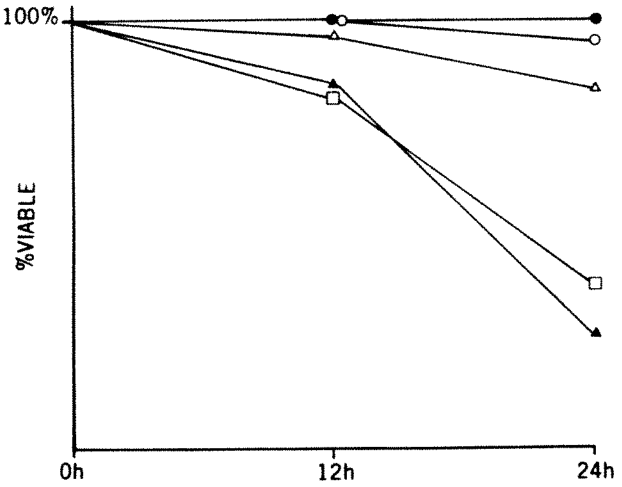

Fig 4. Effect of CAP and analogues on the viability of human bone marrow cells. Cells were incubated with 10^{-4} mol/L drug and without for the intervals shown. Viability was determined by the trypan blue exclusion. Control, ●; CAP, ○; NPAP, △; DHCAP, □; Nitroso-CAP, ▲.

DISCUSSION

The studies described herein clearly demonstrate that two bacterial metabolites of CAP, DHCAP and NPAP, are, like nitroso-CAP, profoundly more cytotoxic than CAP itself. DHCAP was even more toxic than nitroso-CAP, causing total inhibition of CFU-GM growth at the concentration of 10^{-6} mol/L. Furthermore the inhibition of CFU-GM growth was also largely irreversible. All three analogues inhibited DNA synthesis in vitro at a concentration of 10^{-4} mol/L. The apparent difference in the drug concentration required to inhibit DNA synthesis (10^{-4} mol/L) *v* those required to inhibit CFU-GM growth (10^{-6} mol/L to 10^{-5} mol/L) is probably related to differences in experimental conditions, for example, higher cell concentration (5 to 10 × 10^6 cells/mL) and shorter drug exposure (30 minutes) for the DNA synthesis experiment.

On the basis of our studies with nitroso-CAP, we have postulated that bone marrow damage from CAP may be mediated by its nitroreduction intermediates.[1-5] However, in further studies it became clear that nitroso CAP is extremely unstable such that if produced elsewhere it will be rapidly inactivated before reaching its target cell in the bone marrow. The possibility that CAP may be reduced in the marrow itself, thereby generating the toxic nitroso derivative in situ, is unlikely under normal conditions, since significant reduc-

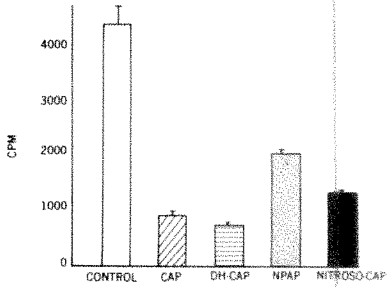

Fig 5. Effect of CAP and analogues on the incorporation of tritiated thymidine by human bone marrow cells. CAP 2×10^{-3}, DHCAP 10^{-4}, NPAP 10^{-4}, nitroso-CAP 10^{-4}. Each column is the result of duplicate assays ± SD.

Table 3. Effect of CAP and Analogues on Rabbit Bone Marrow Mitochondrial Protein Synthesis

Drug	CPM	% Inhibition
0	516 ± 22	—
CAP	103 ± 7	80
Nitroso-CAP	305 ± 10	41
NPAP	321 ± 6	38
DHCAP	358 ± 12	25

Drug concentration 10 μg/mL. Each number represents mean of ten samples, ± SEM.

tion of CAP by normal human bone marrow in vitro could not be demonstrated.[17] Accordingly, the finding that other metabolites of CAP known to be produced by intestinal bacteria are cytotoxic is extremely important and raises the distinct possibility that DHCAP or other bacterial products may play a decisive role in mediating marrow damage from CAP. This hypothesis becomes even more attractive when one considers the fact that DHCAP is toxic at such low concentrations ($\geq 10^{-7}$ mol/L) and is, unlike nitroso-CAP, relatively stable. Thus when mixed with fresh human blood and liver tissue, nitroso-CAP becomes undetectable within seconds. In sharp contrast, 35% and 65% of DHCAP can be recovered from blood and liver respectively, as determined by high-performance liquid chromatography (HPLC) analysis after 30-minute incubation.[18]

It should be noted that whereas serum CAP concentrations of 15 to 60 μg/mL (5×10^{-5} mol/L to 2×10^{-4} mol/L) are known to be reached following therapeutic doses of the drug,[19] nothing is known about actual or potential serum concentrations of any of the metabolites. The recent development of powerful and highly sensitive HPLC detection/separation techniques[20] for these drugs should prove helpful in this regard.

Evidence has been provided by this laboratory and others that CAP-induced reversible bone marrow suppression results from inhibition by the drug of mitochondrial protein synthesis.[21-24] Furthermore both at the mitochondrial and cellular level the action of CAP is reversible.[2,21] Whereas the three metabolites of CAP examined in this study are more cytotoxic than CAP, they are only half as potent as CAP in inhibiting mitochondrial protein synthesis. This plus the fact that their cytotoxicity is largely irreversible indicate that they exert their action by a mechanism different from that of CAP. Indeed data from previous studies strongly suggest that cytotoxicity from nitroso-CAP results from direct DNA damage.[4,25-27] Thus in micromolar concentrations nitroso-CAP inhibits DNA synthesis, arrests dividing cells in the G_2M phase, causes hydrolysis of isolated DNA in vitro and DNA damage in intact cells. In a recent study[27] it was found that DHCAP also causes DNA single-strand breaks in intact cells at concentrations below 10^{-4} mol/L. This is consistent with its ability to inhibit DNA synthesis in bone marrow, as shown in this study.

Because of its toxic nature and its ability to interact with and damage DNA, we suggested that nitroso-CAP may be a mediator of CAP-induced aplastic anemia in the susceptible host. It would appear from the present study that DHCAP or other similar metabolites of CAP are more likely to serve as mediators of aplastic anemia because of their relative stability and therefore greater accessibility to the bone marrow. Since aplasia from CAP is rare, an underlying individual predisposition must be the determining factor. The predisposed subject may produce greater amounts of toxic bacterial metabolites such as DHCAP possibly because of different intestinal bacterial flora. Alternatively, his bone marrow cells may be more sensitive to the metabolites than those of normal subjects. Sensitivity may be manifested by greater damage to DNA or by the subject's inability to repair the damaged DNA. Clearly further studies are needed to explore these and other possibilities.

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Progenitor Cell Assays Predict Hematopoietic Reconstitution After Syngeneic Transplantation in Mice

By Richard J. Jones, Saul J. Sharkis, Paul Celano, O. Michael Colvin, Scott D. Rowley, and Lyle L. Sensenbrenner

Hematopoietic reconstitution following syngeneic bone marrow transplantation with graded doses of untreated and drug-treated bone marrow was studied in B6D$_2$F$_1$ mice. Granulocyte-macrophage colony-forming units (CFU-GM) and spleen colony-forming units (CFU-S) showed similar in vitro drug sensitivities. Both the speed of hematologic recovery and survival of mice transplanted with untreated or drug-treated bone marrow were directly related to the number of CFU-GM or CFU-S transplanted. Similar hematologic recovery was seen for untreated marrow transplants and treated transplants that had similar CFU-GM or CFU-S content. There is a minimum number of transplanted CFU-GM or CFU-S that allows survival of lethally irradiated mice. This number is present in a marrow transplant containing the equivalent of 5×10^3 untreated cells or producing one to two spleen colonies. There also exists a maximum value for the number of hematopoietic progenitors in a marrow graft, above which the rate of hematologic recovery following transplantation is rapid and no detectable increase in the rate is seen with increasing CFU-GM or CFU-S content. The presence of this maximum value for transplanted progenitors and variations in culture techniques are probably the reasons previous studies have not always shown a correlation between CFU-GM content and hematologic recovery after bone marrow transplantation.

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AUTOLOGOUS bone marrow transplantation (BMT) following high-dose chemoradiotherapy has been shown to be a useful treatment for resistant acute leukemias, non-Hodgkin's lymphomas, and Hodgkin's disease.[1-6] However, a potential obstacle to this procedure is the continued presence of tumor cells in autologous marrow, even when the tumor is absent morphologically. Sharkis et al[7] showed that 4-hydroperoxycyclophosphamide (4HC) could eliminate leukemic cells from suspensions of marrow and tumor and still permit hematopoietic reconstitution. Subsequently, multiple pharmacologic,[8-13] immunologic,[2,14] and physical[15,16] methods to eliminate or "purge" tumor from autologous grafts have been studied extensively both in the laboratory and clinically. The ideal "purging" agent for autologous marrow transplantation is one that gives substantial tumor kill while sparing enough hematopoietic progenitors to allow marrow engraftment. One of the difficulties with studying potential "purging" regimens in the laboratory is the absence of an in vitro test for the ability of infused bone marrow to reconstitute the hematopoietic system of the marrow-ablated host.

Using syngeneic marrow transplantation in lethally irradiated mice as a model for autologous marrow transplantation, we assessed the ability of hematopoietic progenitor assays to predict for hematologic reconstitution. Different concentrations of untreated bone marrow as well as marrow incubated in vitro with four different drugs were tested for granulocyte-macrophage colony-forming units (CFU-GM), spleen colony-forming units (CFU-S), and the ability to rescue lethally irradiated mice. The four drugs, 4HC, vincristine, 5-fluorouracil (5FU), and bleomycin, were studied because they have a wide range of antitumor activity and because they clinically spare the hematopoietic system. For these reasons these drugs hold promise as potential "purging" agents for autologous BMT.

MATERIALS AND METHODS

Animals. Female B6D$_2$F$_1$ mice (Cumberland View Farms, Clinton, TN), 6 to 14 weeks of age, were used for all studies except those performed to confirm donor engraftment. In these studies male B6D$_2$F$_1$ mice were used as bone marrow donors and female B6D$_2$F$_1$ mice were the recipients. The animals were housed in sterile microisolator cages. They were fed acidified water and sterilized laboratory chow ad libitum.

Drugs and drug incubations. The four drugs used, 4HC (synthesized by one of the authors, O.M. Colvin), vincristine (Eli Lilly and Company, Indianapolis), 5FU (Sol Pak Laboratories, Franklin Park, IL), and bleomycin (Bristol Laboratories, Syracuse, NY) were diluted in enriched McCoy's 5A medium immediately before each experiment. The enriched McCoy's 5A medium was prepared according to Pike and Robinson.[17]

Mice were killed by cervical dislocation, and the hind legs were removed. Bone marrow was flushed from the medullary cavities of the femurs and tibias using McCoy's 5A medium in a syringe with a 25-gauge needle. Single cell suspensions were made by continued passage through a 25-gauge needle. The cells were then suspended in enriched McCoy's 5A medium at a concentration of 5×10^6 cells/mL for all incubations with graded doses of drugs. After incubation at 25°C for 60 minutes, the cells were washed three times with chilled enriched McCoy's 5A medium and resuspended in this medium for the assays. Over 90% of the cells always remained viable by trypan blue dye exclusion following treatment with any of the four drugs.

Assays. CFU-GM assays were performed using 0.3% agar, 10% L cell-conditioned medium as a source of colony stimulating activity,[18] and between 10^3 and 10^5 marrow cells in enriched McCoy's 5A medium. One milliliter of the bone marrow cell mixture was plated in 35-mm petri dishes. The plates were incubated for seven days in a humidified atmosphere containing 7.5% CO$_2$ at 37°C. Assays were performed in quadruplicate, and the plates were scored using an inverted microscope. A colony was considered to be an aggregate of 50 or more cells.

For the CFU-S and hematopoietic reconstitution studies, the mice

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received 11.0 Gy of total body irradiation (TBI) from a dual ^{137}Cs source at a dose rate of 1.18 Gy/min. The mice were then inoculated with the bone marrow via the tail vein. CFU-S were assayed by the procedure of Till and McCulloch.[19] Briefly, mice were sacrificed at day 7 or 12, the spleens were removed, and all macroscopic colonies (small, medium, and large) were counted after fixing the spleens in Carnoy's solution (60% ethanol, 30% chloroform, and 10% acetic acid). Colonies from random spleens were counted by three different observers, and results were similar. Both day 7 and day 12 CFU-S were performed on eight mice receiving enriched McCoy's 5A medium without any bone marrow cells. No spleen colonies were seen. Mouse survival after 11.0 Gy of lethal TBI and BMT was defined as survival for greater than 60 days. In each reconstitution experiment four irradiated mice received no bone marrow and served as radiation-only controls. All radiation control mice died, usually between days 10 and 14. For sequential neutrophil counts in these mice, the retro-orbital sinus was punctured with a heparinized microhematocrit tube. An attempt was made to limit the blood loss to less than 0.2 mL. WBC counts were determined using an automated cell counter (Coulter Electronics, Hialeah, FL) or by hemocytometer if the count was less than 10^3 cells/mm^3. Differential counts were performed on Wright's stained blood smears, and the absolute neutrophil count was calculated from the total WBC count and the percentage of neutrophils.

All data points were the mean of two to four separate experiments performed in quadruplicate. The number of CFU obtained from assaying 10^5 untreated bone marrow cells was established for each individual experiment and was taken as 100% recovery unless otherwise specified. The results of all transplant studies for survival were the combination of two to five separate experiments. Each individual experiment utilized four to five mice per point. Statistical comparisons were done by simple linear regression analyses with calculation of Pearson's correlation coefficients.

Y chromosome determination by Southern blot analysis of DNA samples. Standard procedures were used in the preparation and quantitation of DNA samples of bone marrow cells.[20] Aliquots of DNA were digested overnight with BamH1 in conditions suggested by the manufacturer (Bethesda Research Laboratories, Gaithersburg, MD). DNA was electrophoresed through 1% agarose gel and transferred to nylon membrane (Gene Screen, New England Nuclear, Boston). Prehybridization and hybridization were performed at 60°C. The membranes were probed with a Y chromosome-specific fragment[21] that was kindly provided by Edward Palmer. Plasmid pY2 was digested with EcoR1 and Sal1. The insert was isolated by agarose gel electrophoresis and purified by electroelution.[20] Radiolabeling with [^{32}P]dCTP (Amersham 3,000 Ci/mmol) was performed by random priming with Klenow DNA polymerase to a specific activity of 0.5 to 1.0 × 10^9 cpm/μg DNA.[22] Washing of filters at high stringency (0.1 × SSC, 0.1% sodium dodecyl sulfate [SDS] at 65°C) resulted in no detectable signal in female DNA samples.

To determine the relative contributions of host- and donor cell-derived material in each DNA sample, the experimental DNA (5 μg total/lane) was blotted in parallel with DNA (5 μg total/lane)) from known mixtures of male and female bone marrow cells as described by Lemischka et al.[23] Filters were probed with BamH1 fragment of mouse c-myc,[24] an autosomal probe, to correct for DNA loading differences and densitometric scanning of lanes was performed.

RESULTS

Syngeneic bone marrow grafts containing between 10^3 and 10^5 untreated nucleated cells were assayed for CFU-GM, CFU-S, and the ability to rescue lethally irradiated mice. Table 1 shows that a linear relationship exists between the number of cells assayed and the number of CFU-GM and CFU-S obtained over the entire range of untreated marrow cells assayed. The results of day 7 and day 12 CFU-S from untreated marrows were the same. Table 1 also shows that the transplantation of only 5 × 10^3 untreated syngeneic marrow cells was able to rescue $^{14}/_{16}$ lethally irradiated mice. The number of CFU-GM observed with assaying 5 × 10^3 untreated marrow cells was 5.4% ± 0.6% of that found for 10^5 untreated cells, and the CFU-S recovery was 7.0% ± 2.6% or 1.9 ± 0.5 actual spleen colonies. The mean number of progenitors observed from all of the experiments was 101 ± 4 CFU-GM and 28 ± 0.6 CFU-S for 10^5 untreated marrow cells assayed.

To verify that the hematopoietic reconstitution following transplantation with 5 × 10^3 bone marrow cells was actually of donor origin, lethally irradiated female $B6D_2F_1$ mice were transplanted with 5 × 10^3 marrow cells from male $B6D_2F_1$ mice. A total of $^{17}/_{24}$ mice from three separate experiments survived. Two additional mice from each experiment were sacrificed at day 21 after transplantation for Y chromosome analysis of bone marrow by the Southern blot analysis of DNA, using a Y-specific DNA fragment.[21] All six mice appeared to be completely reconstituted by donor-derived cells. Southern blots from three of these mice are shown in Fig 1. In addition, two female mice were sacrificed for DNA analysis of bone marrow cells 9 months after BMT with 5 × 10^3 male bone marrow cells. Bone marrow from both mice still appeared to be completely donor derived.

Studies of the hematopoietic function of murine syngeneic marrow grafts after in vitro incubation with 4HC, vincristine, bleomycin, and 5FU were then performed. Graded doses of the four drugs were incubated with 10^5 bone marrow cells at 25°C for one hour. Figure 2 shows the CFU-GM and CFU-S results. The fractional recoveries (log) for CFU-GM and CFU-S are again similar, and both are significantly linearly related to the dose of 4HC, vincristine, or bleomycin. There was no difference in the numbers of spleen colonies recovered on day 7 or 12 from marrow incubated with graded

Table 1. CFU-GM and CFU-S Recovery With Survival of Mice After Transplantation With Graded Concentrations of Untreated Bone Marrow

No. of Marrow Cells	10^3	5 × 10^3	10^4	5 × 10^4	10^5
CFU-GM recovery (%)	1.2 ± 0.7	5.4 ± 0.6	9.8 ± 0.5	54.3 ± 1.9	100 ± 1.7
CFU-S recovery (%)	0	7.0 ± 2.6	10.4 ± 1.2	60.4 ± 5.4	100 ± 3.2
No. of CFU-S observed	0	1.9 ± 0.5	2.9 ± 0.6	16.9 ± 2.4	28 ± 0.6
Survival of mice	0/8	14/16	8/8	8/8	8/8

Data are expressed as mean ± SEM. The number of CFU obtained from 10^5 cells was taken as 100%.

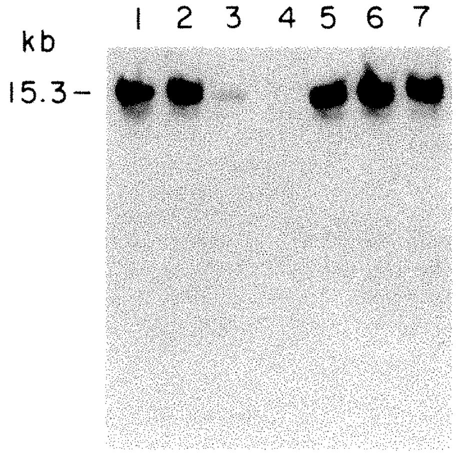

Fig 1. Y chromosome analysis of bone marrow by the Southern blot analysis of DNA using a Y-specific DNA fragment. Lanes 1 to 4 show DNA from mixtures of normal male and female bone marrow cells: lane 1, 100% male; lane 2, 50% male, 50% female; lane 3, 10% male, 90% female; and lane 4, 100% female. Lanes 5 to 7 show DNA of bone marrow from three female mice transplanted with 5×10^3 male bone marrow cells from three separate experiments. The mice were sacrificed on day 21 after transplantation.

doses of these three drugs. However, there were many more spleen colonies seen on day 12 than on day 7, with bone marrow incubated with 5FU. In fact, no spleen colonies were obtained on day 7 from 10^5 marrow cells treated with doses of 5FU greater than 60 μg/mL, despite getting an 8.7% ± 0.9% and a 6.0% ± 0.8% recovery of day 12 CFU-S with 5FU doses of 80 μg/mL and 100 μg/mL respectively. Figure 2 D shows that the same significant linear relationship exists between CFU-GM, day 12 CFU-S, and the in vitro incubation dose of 5FU, as is seen with the other three drugs. No increase in moderate-to large-sized spleen colonies was seen after day 12 for untreated marrow or marrow treated with any of the four drugs. However, a "second wave" of many minute but visible spleen colonies appeared on days 13 to 14 with all syngeneic marrow transplants, as others have described.[25] Table 2 shows that the highest incubation doses of drugs that allowed predictable engraftment by 10^5 marrow cells were again associated with approximately a 5% recovery of both CFU-GM and CFU-S or a recovery of one to two spleen colonies. This is the same recovery seen with assaying 5×10^3 untreated marrow cells.

4HC incubations under the above conditions were also performed using 10^4 and 10^6 marrow cells. Results are shown in Table 3. The highest incubation dose of 4HC that allowed survival of the lethally irradiated mice was 10 μg/mL for 10^4 marrow cells and 80 μg/mL for 10^6 cells. The former condition produced a 48% ± 6% recovery of CFU-GM and a 36% ± 1% recovery of CFU-S. The latter condition produced a 0.5% recovery for both progenitors. Both of these conditions produced one to two spleen colonies. The percent recoveries of CFU-GM and CFU-S for drug-treated bone marrow were independent of the number of marrow cells assayed. Engraftment of lethally irradiated mice by syngeneic marrow incubated with drugs was directly related to the number of transplanted CFU-GM or CFU-S. This equals the product of the pretreatment bone marrow CFU-GM or CFU-S content and their fractional recoveries after treatment. Again, survival of the mice occurred when the CFU-GM or CFU-S content of the transplant equalled that of 5×10^3 untreated bone marrow cells. Combining the survival results from all the different conditions tested (Tables 1, 2, and 3) reveals that 132 mice received transplants that produced at least one spleen colony. Of these 132 mice, 124 survived. Transplants producing less than one spleen colony allowed only $^{10}/_{92}$ mice to survive.

To assess the kinetics of hematologic recovery, neutrophil counts on days 7, 10, 14, and 21 after syngeneic BMT were performed. Figure 3 shows the circulating neutrophil counts on days 7, 10, and 14 after marrow transplantation with graded doses of untreated bone marrow. Mice receiving at least 10^6 marrow cells had normal neutrophil counts (greater than 2,000 absolute neutrophils) by day 14, and all surviving mice had normal neutrophil counts by day 21. The speed of neutrophil count recovery increased with increasing the number of transplanted cells up to a maximum of 5×10^6 cells. There were no differences in the recovery kinetics for marrow inoculums of 5×10^6, 10^7, or 2×10^7 cells. All mice developing 100 or more neutrophils/μL by day 10 survived. Interestingly, even mice transplanted with 10^3 marrow cells developed a small number of circulating neutrophils by day 10, although all these mice eventually died. No radiation control mice developed circulating neutrophils. Table 4 shows the results from periodic sampling of circulating neutrophil counts after transplanting 10^5 marrow cells that had been incubated in vitro with graded doses of 4HC. Untreated bone marrow transplants and 4HC-treated transplants, which contained similar numbers of CFU-GM or CFU-S, produced similar neutrophil recoveries. RBC recovery was parallel to neutrophil recovery for all of the conditions studied (data not shown). However, the nadir and ultimate recovery of the RBC counts lagged slightly behind the neutrophil counts.

DISCUSSION

Although CFU-GM assays have been used previously to study the effects of potential "purging agents" on the hematopoietic function of bone marrow,[8,9,12,13,16] there was no clear evidence that this assay correlated with hematopoietic recovery. While some series of patients receiving "unpurged" BMTs have suggested a relationship between the number of CFU-GM infused and the speed of hematopoietic recovery,[26-29] others have not.[30,31] Earlier reports from this institution also indicated that the CFU-GM assay could not be used to predict hematologic recovery after 4HC "purged" autologous marrow transplantation, since marrows treated with 100 μg/mL of 4HC allowed engraftment despite no recovery of CFU-GM.[3,10] We have subsequently found that the hematologic recovery of lethally irradiated mice receiving BMTs was directly related to the number of CFU-GM or CFU-S transplanted. Untreated and drug-treated bone marrow grafts with similar CFU-GM and CFU-S content produced similar hematologic recovery in transplanted mice, despite there being large differences in the numbers of transplanted

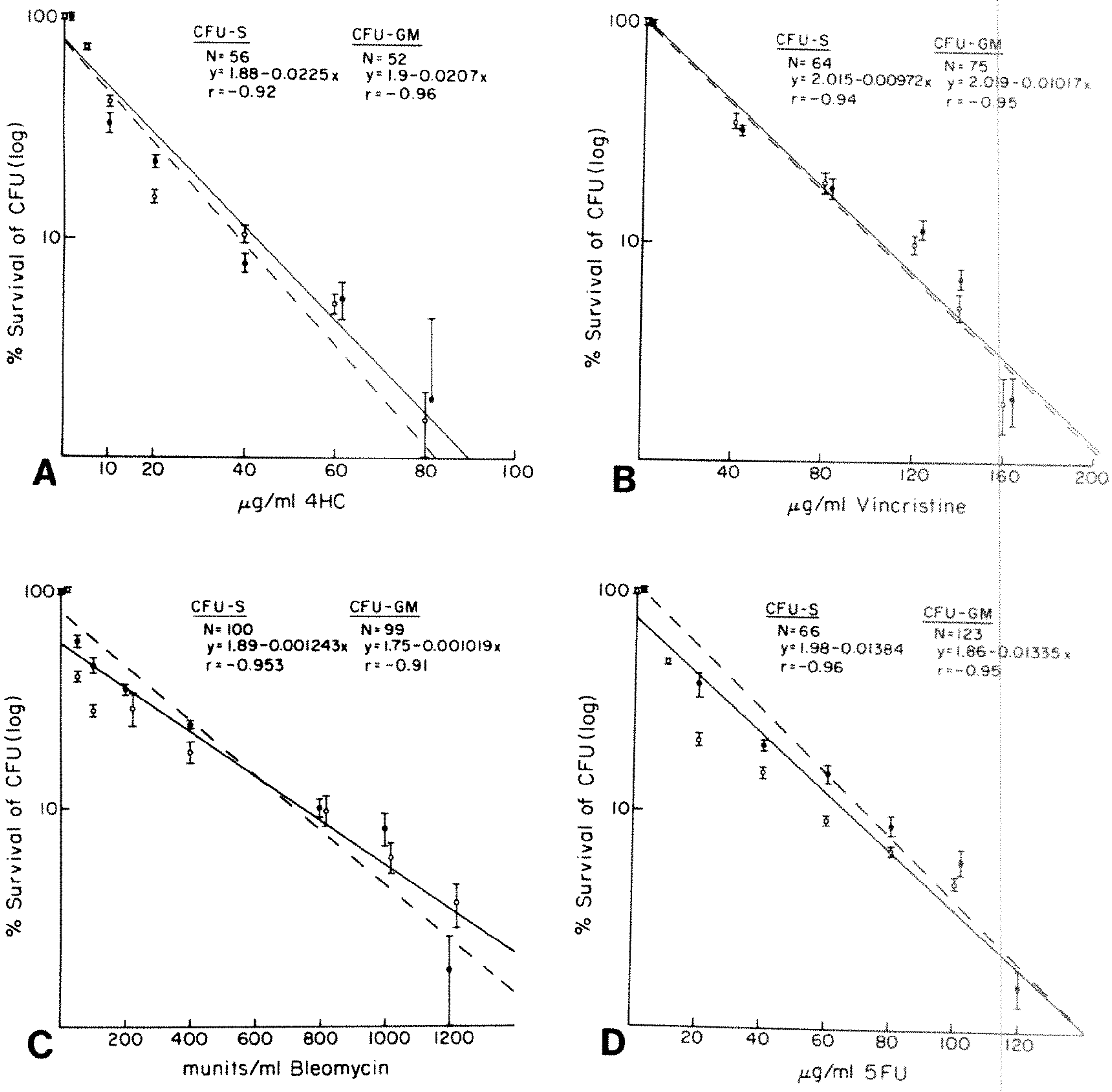

Fig 2. Relation of percent survival of CFU-GM (○—○) and CFU-S (●—●) for 10^5 marrow cells incubated in vitro with 4HC (A), vincristine (B), bleomycin (C), and 5FU (D). (A through C) CFU-S results are the same on both days 7 and 12. (D) Only day 12 CFU-S are shown (see text). Each data point represents the mean ± SEM of two to four experiments performed in quadruplicate. Pearson's correlation coefficients (r) for all linear regressions are significant ($P < 10^{-6}$).

bone marrow cells. Also, two recent series of autologous marrow transplants after "purging" with 4HC or monoclonal antibodies (MoAbs) have shown a positive correlation between CFU-GM results and hematopoietic recovery.[32,33]

There are at least two factors that explain the differing findings regarding the predictive value of the CFU-GM assay. The first factor concerns the conditions used for culturing CFU-GM. With the culture conditions used in this study, utilizing 15% fetal calf serum (FCS), we were able to reproducibly detect at least a 1% CFU-GM recovery after

Table 2. CFU-GM and CFU-S Recovery With Survival of Mice After Transplantation With 10^5 Bone Marrow Cells Incubated With Graded Concentrations of Drugs

	4HC(µg/mL)		Vincristine (µg/mL)		5FU(µg/mL)		Bleomycin (mU/mL)	
Drug Concentration	60	80	140	160	100	120	800	1200
CFU-GM recovery (%)	5.1 ± 0.6	1.8 ± 0.5	5.2 ± 0.9	1.9 ± 0.6	4.8 ± 0.3	1.6 ± 0.3	9.7 ± 0.9	3.7 ± 0.8
CFU-S recovery (%)	5.4 ± 1.3	1.9 ± 2.6	7.1 ± 0.7	2.1 ± 0.5	6.0 ± 0.8	0.9 ± 0.4	9.8 ± 0.9	1.8 ± 0.8
No. of CFU-S observed	1.5 ± 0.4	0.6 ± 0.6	2.0 ± 0.4	0.6 ± 0.04	1.7 ± 0.2	0.2 ± 0.1	2.2 ± 0.64	0.5 ± 0.1
Survival of mice	10/10	0/10	12/12	6/12	15/16	1/16	25/28	0/20

CFU-S results are the same on both day 7 or 12 for 4HC, vincristine, and bleomycin. CFU-S results with 5FU are only for day 12. Data are expressed as mean ± SEM.

Table 3. CFU-GM and CFU-S Recovery With Survival of Mice After Transplantation With Graded Concentrations of Bone Marrow Incubated With Graded Doses of 4HC

No. of Marrow Cells	10^4		10^5		10^6	
Drug Concentration (μg/mL)	10	20	60	80	80	100
CFU-GM recovery (%)	48 ± 6.4	11 ± 5.0	5.1 ± 0.6	1.8 ± 0.5	0.54 ± 0.06	0
CFU-S recovery (%)	36 ± 1.0	0	5.4 ± 1.3	1.9 ± 2.6	0.51 ± 0.01	0
No. of CFU-S observed	1.1 ± 0.2	0	1.5 ± 0.4	0.6 ± 0.6	1.5 ± 0.2	0
Survival of mice	7/8	2/8	10/10	0/10	7/8	1/8

Data are expressed as mean ± SEM.

drug incubation. Similar culture conditions with human marrow, as were used in previous reports of "purging" with 4HC from this institution,[3,10] however, permitted only a 5% to 10% CFU-GM recovery to be seen. Nutrient enrichment in our agar culture using 30% FCS, 1% bovine serum albumin, and 10^{-4} mol/L 2-mercaptoethanol has allowed us to see nearly a tenfold increase in CFU-GM cloning efficiency without a change in CFU-GM drug sensitivity (unpublished data). We are now able to culture CFU-GM from all marrows "purged" with 100 μg/mL of 4HC. Utilizing similar enriched culture conditions, Rowley et al[33] were able to find an average CFU-GM recovery of 4.5% (range 0.07% to 23%) after "purging" autologous marrow grafts with 100 μg/ml of 4HC. These much smaller fractional recoveries of CFU-GM permitted us to establish the correlation between CFU-GM and hematopoietic recovery, while the previously used culture conditions could not.

Second, there appears to be a maximum number of transplanted progenitors that allows the correlation with speed of hematologic recovery to be seen. We found no detectable difference in the kinetics of hematopoietic recovery in mice receiving 5 × 10^6 untreated marrow cells or greater, although the kinetics of hematologic recovery were strongly correlated with the number of cells or CFU-GM transplanted below this level. The CFU-GM content averaged over 2 × 10^5 CFU-GM/kg, with all patients receiving at least 5 × 10^4 CFU-GM/kg, in the marrow transplants from the studies that showed no correlation between CFU-GM and hematopoietic reconstitution.[30,31] Conversely, in four of the studies showing a relationship, an average of only 3 × 10^3 to 4 × 10^4 CFU-GM/kg were transplanted per patient.[26,28,29,33] Our present animal data and the results of these clinical studies taken collectively suggest that there exists a maximum value for the number of hematopoietic progenitors transplanted, above which the rate of hematologic recovery following BMT is rapid and no detectable increase in the rate can be seen with increased CFU-GM content. However, below this maximum value hematopoietic recovery correlates with the CFU-GM content of transplanted marrow.

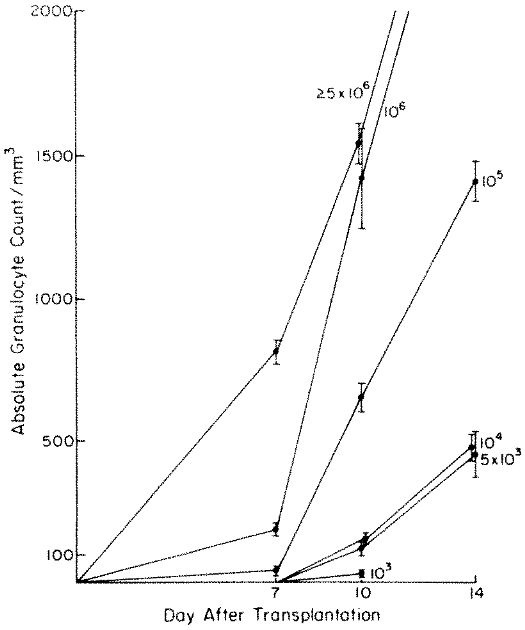

Fig 3. Kinetics of circulating neutrophil recovery after transplantation of graded concentrations of untreated bone marrow cells. There were no differences in results for marrow transplants containing 5 × 10^6, 10^7, or 2 × 10^7 cells. All mice transplanted with 5 × 10^3 or more cells survived. Those transplanted with 10^3 cells died between days 10 and 14. Each data point represents the mean ± SEM of eight mice from two separate experiments.

Hematopoietic reconstitution occurred in most mice receiving a bone marrow graft containing at least 5 × 10^3 untreated bone marrow cells. This is consistent with the findings of Boggs et al,[34] who found that the inherited macrocytic anemia of W/W^v mice could be cured with 10 × 10^3 +/+ bone marrow cells. Mice appeared to require at least 100 circulating neutrophils/μL by day 10 after BMT to survive. Although all mice that received either 10^3 untreated marrow cells or 10^5 cells incubated with 80 μg/mL of 4HC died, they still developed some circulating neutrophils (but less than 100 μL) by day 10. This suggests that early engraftment may be developing but not quickly enough to prevent death from the complications of aplasia. Therefore hematopoietic reconstitution and survival in mice after BMT may be related to the speed of hematologic recovery. Although there was a clear minimum number of CFU-GM

Table 4. Circulating Neutrophil Counts/μL After Transplantation of 10^5 Marrow Cells Incubated In Vitro with 4HC

Dose of	Day After Transplantation			
4HC (μg/mL)	7	10	14	21
20	0	208 ± 50	1,070 ± 194	2,550 ± 141
40	0	149 ± 10	399 ± 46	2,333 ± 72
60	0	109 ± 22	411 ± 60	2,096 ± 83
80	0	33 ± 15	—	—

All mice transplanted with 10^5 marrow cells incubated with 80 μg/mL of 4HC died between days 10 and 14. Data are expressed as mean ± SEM.

or CFU-S needed to allow survival of the transplanted mice, it is possible that mice receiving much smaller numbers of progenitors could survive with increased support during aplasia.

We found no difference between the numbers of day 7 and day 12 CFU-S after transplantation with untreated marrow or marrow treated with 4HC, vincristine, or bleomycin. However, we did find that the numbers of day 12 spleen colonies were increased compared to day 7 after in vitro incubation of bone marrow with 5FU. Others have also reported that while the numbers of CFU-S remained constant between day 8 and day 14 after transplantation with untreated bone marrow, the numbers of CFU-S following transplantation with 5FU-treated marrow increased over this time.[35-38] Hodgson and Bradley[35] proposed that this increase in spleen colony numbers with time after transplantation of 5FU-treated marrow may be due to noncycling stem cells that are more resistant than committed progenitors to 5FU. However, we found that day 12 CFU-S have the same sensitivity to 5FU as the committed hematopoietic progenitor, CFU-GM. This finding may suggest that the delayed extra spleen colonies that appear after 5FU treatment occur as CFU-S repair sublethal damage done by 5FU, as was proposed by Rosendaal et al.[36]

We have found, as have others,[39,40] that the CFU-GM and CFU-S show similar sensitivities to drugs. It has been reported that the cells that form both of these colonies are relatively mature progenitors and thus are not the cells responsible for marrow engraftment.[35,41] Recent reports have also described a primitive, multipotential progenitor (the blast colony-forming cell) that is more resistant than CFU-GM to 4HC.[42,43] These reports make it difficult to explain the ability of CFU-GM and CFU-S to predict hematopoietic reconstitution after BMT. One possibility is that although the CFU-GM and CFU-S are not the cells responsible for engraftment, they could still predict the response of the stem cell to manipulations if their quantities and drug sensitivities remained proportional to those of the stem cell. Evidence against this hypothesis is that reconstitution in our study was related to the same degree of CFU-GM inhibition after in vitro incubation of the marrow with four different drugs. It is unlikely that drug sensitivity differences between the stem cell and CFU-GM would be the same for all four drugs. In fact, Sahovic et al[43] showed that although the primitive multipotential progenitors were more resistant than CFU-GM to 4HC, there were no differences in sensitivities to the alkylating agent, phenyl-keto-phosphamide.[43] A more likely explanation for the ability of CFU-GM and CFU-S to correlate with hematopoietic reconstitution is that there may be two phases of engraftment after BMT. Early hematopoietic recovery could be due to mature committed progenitors, while primitive pluripotential progenitors would be responsible for later, sustained engraftment. If this were the case, initial engraftment would be expected to correlate directly with committed progenitors like the CFU-GM.

Both the CFU-GM and CFU-S content of bone marrow, therefore, predict hematopoietic recovery after syngeneic transplantation in mice. This is true even for marrow treated with drugs like 4HC, which may have differential activity toward committed and multipotential hematopoietic progenitors. A minimum number of transplanted CFU-GM or CFU-S is needed to allow survival of lethally irradiated mice. There is also a maximum number of transplanted progenitors above which the correlation between progenitor content in the transplant and speed of hematologic recovery is not seen. Obviously the true effectiveness of any purging regimen for autologous BMT can only be assessed from clinical trials. However, the CFU-GM assay should prove to be a useful technique to assess the effect of potential "purging" agents on hematopoietic function.

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Human Leukemic B Cell Activation: Functional Consequence of Membrane IgM Interaction With Anti-IgM Ligand Is an Alterable Cell Characteristic

By Patricia Mongini, Clare Blessinger, Stephanie Seremetis, Robert Winchester, and Steven Rudich

A functional study of several human malignant B cell populations has indicated that occasional leukemic clones are extraordinarily sensitive to signal transduction through membrane IgM. One isolated hairy cell leukemia (HCL) with low background DNA synthesis was stimulated to significant levels of DNA synthesis when cultured with high (100 μg/mL) concentrations of soluble anti-IgM ligands. In contrast to the activation of normal peripheral blood polyclonal B cells, this DNA synthesis was completely independent of accessory T cell factors. Although the HCL clone could also be induced to enter S phase by incubation in media supplemented with only activated T cell supernatant, culture of the clone with activated T cell supernatant plus anti-IgM Ab resulted in DNA synthesis that was significantly less than that induced by either activator alone. Factor(s) in T cell supernatant appear to modulate the leukemic clone so that the binding of ligand to membrane IgM is perceived as an inhibitory rather than a stimulatory signal for DNA synthesis. In terms of Ig Fc independence and low ligand dose requirements, anti-IgM-mediated inhibitory signal transduction in the T cell supernatant-activated HCL clone was found to mimic anti-IgM mediated suppression of the spontaneous DNA synthesis of an alternative HCL clone. The functional results suggest that the type of signal transduced anti-Ig ligands may reflect differences in the activation state of receptive leukemic B cells.

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RECENT STUDIES suggest that the phenotypic diversity of B cell leukemias and lymphomas corresponds to diversity within the pool of normal B lymphocytes.[1,2] Thus, the membrane antigen expression of distinct malignant B cell populations appears to reflect the pattern of cell surface marker acquisition during B lymphocyte maturation[3-5] and many functional characteristics of malignant B cells likely mimic those of defined subsets of normal B lymphocytes.[2] It has been suggested that the phenotype of normal B cells at each stage of differentiation will be best elucidated by studies of clonal populations of malignant B cells of the corresponding differentiation state. This is supported by the fact that many features of malignant cells initially thought to be aberrant were ultimately shown to be reflective of those of normal, but at the time undiscovered, cells or functions.[2,6-10]

One particular functional feature of normal polyclonal B cells that has been mimicked in occasional leukemia or lymphoma cell populations is sensitivity to stimulatory or inhibitory signal transduction by ligands that cross-link membrane IgM (mIgM). The relatively infrequent examples of mIgM-mediated stimulatory signal transduction that result in enhanced in vitro DNA synthesis of malignant B cell clones are thought to represent an activation phenomenon characteristic of mature resting B cells.[11,12] In contrast, signaling through membrane IgM that results in inhibition of DNA synthesis has been thought to represent a form of tolerogenic signal transduction most clearly manifest in normal immature B lymphocytes.[13-16]

We have attempted to identify malignant B cell populations that will facilitate clonal studies of the mechanisms of stimulatory and inhibitory signal transduction following ligand binding to mIgM. This has involved a screen of diverse membrane IgM-positive B cell leukemias for responsiveness to soluble anti-IgM antibody following in vitro culture. In this report we document examples of positive and negative signal transduction by soluble anti-IgM antibodies in a form of leukemia thought to represent a malignancy of mature B lymphocytes, ie, hairy cell leukemia (HCL).[3,4] We further demonstrate the unique finding that soluble factors from activated T cells can modulate the responsiveness of one of the leukemic cell populations to anti-Ig ligands such that an inhibitory rather than a stimulatory signal is transduced following membrane Ig cross-linking. These findings indicate that the nature of membrane IgM-mediated signal transduction in certain B cells with a mature functional phenotype can be changed following exposure to certain T cell factors. This modulated response may mimic that of an infrequent subpopulation of normal mature B lymphocytes, which is masked in activation studies using polyclonal B cells. Both the heterogeneity in responsiveness to membrane Ig binding ligands observed with these leukemic populations and the ability to change the functional phenotype of a given leukemic clone may have important implications for the use of anti-Ig ligands in immunotherapy.

MATERIALS AND METHODS

Leukemic and normal B cells. The malignant B cell specimens used in this study were obtained from Dr Janet Cuttner of Mount Sinai School of Medicine. Patients were afflicted with either chronic lymphocytic leukemia (CLL), poorly differentiated lymphocytic lymphoma (PDLL) or HCL. Neoplastic cells were classified as HCL based on morphology and tartrate resistant acid phosphatase positivity.[17] Patient samples consisted in 12 cases of peripheral blood (PB) or leukophoresis specimens and in one case (SAP) of a spleen specimen. In all instances, specimens were obtained before therapy. Cells were stored in the vapor phase of liquid nitrogen until use. The cell populations selected for functional studies consisted of those with relatively high leukemic cell counts to minimize effects of contaminating cells as well as to insure availability of substantial numbers of cells for extended study. Before being functionally tested for receptivity to anti-IgM Ab-mediated signaling, the cell popula-

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tions were screened for expression of membrane IgM by visual immunofluorescence with HB-57 anti-human IgM monoclonal antibody (MoAb)[18] and fluorescein-conjugated goat F(ab)2 anti-mouse Ig.

Normal B cell and T cells preparations were obtained from normal volunteer human PB by twice rosetting Ficoll-Hypaque isolated cells with neuraminidase-treated sheep RBCs, as previously described.[18] Sheep RBCs were removed from the rosette-positive (ER-positive) cells by lysis with ammonium chloride in Tris buffer.

Cytotoxic depletion of monocytes and T cells from leukemic cell preparations. Potential contaminating monocytes and T cells were removed from leukemic cell populations by treatment with the pan-T cell MoAbs, BW 264/50 (CD7) and T3/2ad 2A2 (CD3),[19] both of IgM isotype, the pan-monocyte MoAbs, MθS-1 and MθP-9 (Leu M3)[20] of IgG2a and IgG2b isotypes, respectively, and complement (C). Briefly, the procedure entailed incubating leukemic cells at 10×10^6/mL in either medium (see culture medium formulation), or medium supplemented with concentrations of the pan T MoAbs and/or the pan-monocyte MoAbs four times the concentration shown to be saturating for normal PB T cells or monocytes, respectively, by previous immunofluorescence analyses. Following 45 minutes of incubation with occasional shaking, the cells were centrifuged and resuspended in a 1:5 dilution of low background cytotoxicity rabbit serum (Pel-Freez, Rogers, AR) as the source of C. After incubation at 37°C for 45 minutes, the cells were washed and placed in culture at a concentration of 2×10^5/200 μL. The effectiveness of this procedure at depleting possible contaminating T cells and monocytes, which were virtually undetectable in the original leukemic cell preparations by immunofluorescence staining, was evidenced by 68% specific cytotoxicity of normal PB cells with the pan-T cell MoAb cocktail + C and a 52% cytotoxicity of PB cells from a patient with myeloproliferative disease with the pan-monocyte MoAb cocktail + C. This latter cell population had been shown to be 58% positive for MθS-1 and MθP-9 by visual immunofluorescence microscopy while normal PB cells routinely contain 50% to 70% T cells by immunofluorescence analysis.

Immunofluorescence. Binding of MoAbs to the surface of leukemic cells was determined by indirect immunofluorescence assays with the fluorescence-activated cell sorter, FACS IV (Becton Dickinson, Mountain View, CA) as described.[18,20,21]

The identity, specificity, and murine isotype of the MoAbs used for the immunofluorescence assay are as follows: HB-57 anti-human IgM (IgG1) obtained from American Type Culture Collection (ATCC)[18]; 4E5 anti-human IgD (IgG1)[18]; HB-61 anti-human kappa light chain (IgG1) from ATCC[18]; 4C6 anti-lambda light chain (IgG3)[18]; MθP-9 (Leu M3) anti-monocyte (IgG2b)[20]; 89b1 anti-pan T cell (IgG1)[22]; Leu 1 (IgG2a) with specificity for all T cells and a subset of B cells[7] from Becton Dickinson, Sunnyvale, CA; 91d4 (Leu 1-like) (IgG1)[7,22]; FMC7 anti-limited B cell (IgM), a gift of Dr Heddy Zola[23,24]; 4F2 with specificity for an activation marker on lymphocytes and monocytes (IgG2a) from ATCC[25]; 5E9 anti-transferrin receptor (IgG1) from ATCC[26]; S157 monomorphic anti-DR (IgG2a)[27]; B1 (IgG2a) and B4 (IgG1) each anti-pan B cell[28,29]; B2 anti-limited B cell (IgM)[30,31]; J5 anti-CALLA (IgG2a)[32]; and PC-1 anti-plasma cell (IgM).[33] The latter five Abs, ie, B1, B2, B4, J5, and PC-1 were obtained from participation in the Second International Congress of Leukocyte Antigens. HB-57, 4E5, HB-61, 4C6, 89b1, 91d4, and FMC7 were used in supernatant form for staining. MθP-9, B1, B2, B4, J5, PC-1, and 5E9 were used in diluted ascites form. 4F2, Leu 1, and S157 were used as purified proteins diluted to 50 μg/mL in assay buffer, ie, PBS + 1% bovine serum albumin (BSA) + 0.1% sodium azide. The isotype control Igs consisted of purified myeloma proteins, MOPC-21 (IgG1), UPC-10 (IgG2a), J606 (IgG3), MOPC-195S (IgG2b), and MOPC-104E (IgM) at a concentration of 50 μg/mL in assay buffer. The IgG MoAbs were ultracentrifuged in an airfuge (Beckman Instruments, Palo Alto, CA) at 100,000 × g for 30 minutes, and the IgM MoAbs at 100,000 × g for ten minutes before use.

Reagents for culture. The anti-human IgM murine MoAbs HB-57 and XG9 and the murine IgG myeloma proteins MOPC-21 and MOPC-245, of undefined specificity, were purified from murine ascitic fluid as described elsewhere.[18] F(ab)2 fragments of HB-57 were prepared by pepsin digestion. Briefly, 30 mg of purified MoAb in 0.1 mol/L citrate, pH 3.6, was treated with pepsin (Sigma Chemical Co, St Louis) at 37°C with an enzyme to substrate ratio of 1:60 for one hour. Digestion was terminated by raising the pH >8 by the addition of solid Tris. Potential undigested Ab was removed by passing the digest over Protein-A-Sepharose (Pharmacia) by the method of Ey et al.[34] Protein-A-Sepharose nonbound Ab was vacuum-concentrated and sieved through a Sephacryl S-200 (Pharmacia) gel filtration column. Fractions from a single observed peak were concentrated and analyzed by sodium-dodceyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for purity. Analysis of the HB-57 pepsin-digested Ab in a 5% to 20% gradient gel under nonreducing conditions revealed only one band of molecular weight 125,000 in contrast to the 150,000 mol wt band of intact MoAb. Goat anti-human IgM Ab in F(ab)2 form was the kind gift of Dr Nicholas Chiorazzi.

T cell supernatants used in these studies were derived from cultures of PHA-stimulated mixtures of PB cells of allogeneic individuals as has been described previously.[18] Briefly, each supernatant lot was generated by pooling PB cells from four to ten healthy donors and culturing them at a concentration of 1.5×10^6/mL in 24-well plates in the presence of 2 μg/mL phytohemagglutinin (PHA-P; Difco Laboratories, Detroit). Supernatants were removed after three days of incubation and dialyzed in Iscove's-Hams's supplemented culture medium before being frozen in aliquots at −70°C. The experiments shown in this report were reproduced with three distinct lots of activated T cell supernatants. In some experiments, purified anti-PHA-P Ab (Collaborative Research, Lexington, MA) was added to test for possible effects of contaminating PHA-P. 4β-phorbol 12β myristate 13α acetate (PMA) was obtained from Sigma Chemical Company. Pokeweed mitogen (PWM) was obtained from Gibco Laboratories, Chagrin Falls, OH.

Cell culture. Cells were routinely cultured in 96-well microtiter plates (Flow Laboratories, McLean, VA) at 2×10^5 cells per 0.2 mL culture volume in a humidified 37°C incubator with 5% CO_2. Medium used for culture was a Ham's-Iscove's mixture with 15% fetal calf serum and additional supplements described elsewhere.[18] DNA synthesis was assessed by uptake of an 18-hour pulse of 1 μCi ^{3}H-thymidine (New England Nuclear, Boston). Cultures were harvested from two to eight days after initiation by previously described techniques.[18] The data are shown as the mean ^{3}H-thymidine uptake in three to five replicate cultures ± SEM.

Assay for Ig secretion. Cultures of certain leukemic cells were assessed for Ig secretion by radioimmunoassay (RIA) of culture supernatants. Following culture from d0 to d4(d5) in the presence or absence of anti-IgM MoAb and/or T cell supernatant, cells were washed four times with warm medium while in culture wells and subsequently recultured for an additional three to four days in medium without anti-IgM MoAb or T cell supernatant. The washing and reculture procedure was performed to remove soluble anti-IgM from the first culture period that might interfere with detection of secreted IgM by the RIA. Evidence for the removal of anti-IgM was obtained on observing that the addition of anti-IgM Ab to IgM secreting cultures immediately before the wash and reculture period resulted in no loss in amount of IgM detected by RIA. At the end of the second culture period, the pooled supernatants from quintuplicate cultures were evaluated for the presence of IgM, IgG, and IgA1 antibody by a modification of a solid phase RIA described previously.[35,36] Briefly, polyvinylchloride wells were coated with purified anti-human kappa MoAb at 20 μg/mL in PBS. Serial dilutions of culture supernatant in 1% BSA-containing PBS were then incubated on the coated wells. Various concentrations of purified human IgM kappa, IgG1 kappa, and IgA1 kappa myeloma proteins were also incubated on anti-kappa coated wells. Bound Ig was detected with ^{3}H-labeled MoAb probes to human Ig classes that have been described elsewhere,[36] ^{3}H-HB-57 anti-IgM, ^{3}H-DC anti-pan IgG, and ^{3}H-CBA1C-7B10 anti-IgA1. The amount of Ig of each isotype in culture supernatant was determined by use of standard curves established with the appropriate human Ig myeloma proteins. The thresholds of sensitivity of these RIAs for IgM, IgG, and IgA1, were 4, 31, and 8 ng/mL, respectively.

RESULTS

Identification of two leukemic clones with receptivity to signal transduction by soluble anti-IgM ligand. Membrane IgM-positive malignant cell preparations from five patients with HCL, five patients with CLL, and one patient with PDLL were tested for possible modulation of in vitro DNA synthesis by a murine monoclonal anti-human IgM MoAb (HB-57). This ligand has previously been shown to be mitogenic in soluble form for normal human B lymphocytes.[18] The data in Table 1 indicate that within this leukemic panel, two HCL clones showed striking and distinctive responses to culture with a 100 μg/mL concentration of the soluble anti-IgM Ab. KON leukemic cells, which exhibited no background DNA synthesis, showed very significant increases in ^{3}H-thymidine incorporation in the presence of the ligand. In contrast, LUB cells exhibited a high level of background DNA synthesis that was suppressed, ie, 94% inhibition, by this MoAb. The above responses were not restricted to the use of the HB-57 MoAb. Identical stimulatory and inhibitory phenomena were observed with a polyclonal F(ab)2 preparation of affinity-purified goat anti-human IgM antibody (data not shown). The two HCL clones that manifest responsiveness to anti-IgM ligands were both characterized by an unusual hyperleukocytosis for this form of leukemia. It remains to be determined whether receptivity to anti-IgM mediated signals is characteristic of such forms of this malignancy.

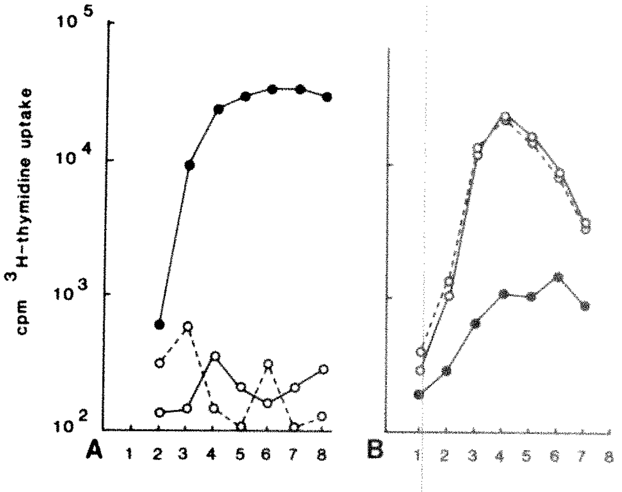

Fig 1. Time course of the positive and negative effects of soluble anti-IgM Ab on DNA synthesis of cultured KON and LUB HCL B cells. Cells were incubated with 100 μg/mL of soluble HB-57 anti-IgM MoAb, control MOPC-245 myeloma protein or medium alone for various intervals. DNA synthesis was assessed by uptake of an 18-hour pulse of 1 μCi ^{3}H-thymidine at the termination of the culture period. The data represent the mean of cpm ^{3}H-thymidine uptake in quintuplicate cultures. O---O, Medium only; O—O, +100 μg/mL MOPC-245; ●—●, +100 μg/mL HB-57 anti IgM.

An extended kinetic analysis of the in vitro DNA synthesis observed with KON and LUB leukemic cells both in the presence and absence of anti-IgM ligand is shown in Fig 1. KON cells stimulated with HB-57 Ab showed maximal and sustained levels of ^{3}H-thymidine uptake from d4-d8 of culture. This DNA synthesis did not result in extensive and prolonged in vitro proliferation, however (data not shown). The spontaneous DNA synthesis characteristic of LUB cells peaked at d4 of culture. This also was a transient phenome-

Table 1. Responses of Membrane IgM-positive Malignant B Cell Populations to Soluble Anti-IgM Antibody

Donor	Leukemic Classification	Cells/mm³	cpm ^{3}H-Thymidine Uptake ($\bar{X}$ ± SEM)		
			Medium	MOPC-21 (MOPC-245)	HB-57 Anti-IgM MoAb
KON	HCL	340,000	353 ± 48	445 ± 99	15,936 ± 1,049
LUB	HCL	262,000	11,147 ± 137	11,350 ± 440	655 ± 43
LIE	HCL	35,400	1,632 ± 183	2,380 ± 166	2,350 ± 56
ZAL	HCL	89,000	255 ± 30	ND	363 ± 22
SAP	HCL	Spleen	2,131 ± 324	2,234 ± 522	4,212 ± 350
LAN	CLL	324,000	219 ± 2	161 ± 6	175 ± 11
SCH	CLL	66,800	94 ± 7	235 ± 22	157 ± 6
DAV	CLL	506,000	470 ± 123	732 ± 17	143 ± 42
WHI	CLL	80,000	399 ± 44	ND	753 ± 423
BRE	CLL	330,000	118 ± 12	ND	140 ± 44
SCH	PDLL	149,000	395 ± 61	ND	259 ± 24

Populations of peripheral blood cells (exception = SAP spleen) of either HCL, CLL, PDLL classification were characterized as positive for mIgM by visual immunofluorescence microscopy and cultured at 2 × 10^5 per well in the presence of 100 μg/mL HB-57 anti-IgM MoAb or MOPC-21 (MOPC-245) myeloma protein as a murine IgG1 control. After 48 to 72 hours, the cultures were pulsed with 1 μCi per well ^{3}H-thymidine and cultures continued for an additional 18 hours. Cells were harvested onto glass filters and counts per minute of ^{3}H-thymidine incorporation was determined by scintillation counting techniques.

Abbreviation: ND, not done.

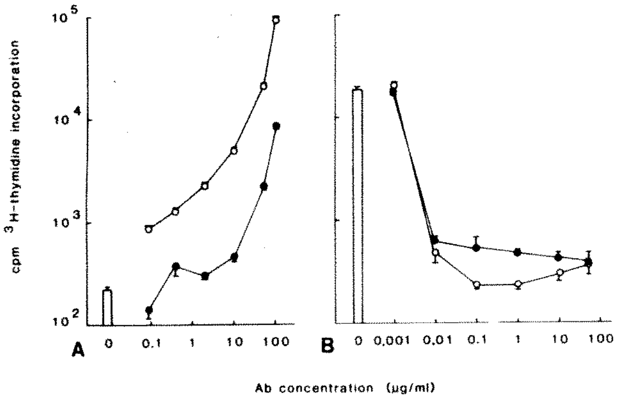

Fig 2. Comparison of the response of KON and LUB leukemic B cells to various concentrations of intact (●) or F(ab)$_2$ fragments (○) of HB-57 anti-IgM MoAb. The incorporation of an 18-hour pulse of ^{3}H-thymidine was measured on d5 (KON cells) or d4 (LUB cells).

non in that cell lines of these cells could not be established without transformation by Epstein Barr Virus (data not shown). LUB cells cultured in the presence of anti-IgM ligand showed suppressed levels of DNA synthesis throughout the one to seven days of culture (Fig 1), which did not reflect a ligand-induced decrease in viability (data not shown).

As indicated in Fig 2, a striking difference was noted in dose requirements for stimulatory signal transduction in KON cells *v* inhibitory signal transduction in LUB cells. While >95% inhibition of DNA synthesis was achieved by concentrations of HB-57 ligand as low as 10 ng/mL, stimulation of DNA synthesis in KON cells was best achieved by the highest concentration of Ab tested, ie, 100 µg/mL. The Fc portion of the antibody ligand did not contribute to the transduction of inhibitory signals in LUB cells as indicated by the identical inhibitory effects of intact and F(ab)2 forms of HB-57 anti-IgM antibody (Fig 2). However, the presence of the Fc portion of the ligand did dampen its stimulatory effects for KON cells since F(ab)2 fragments consistently induced higher levels of ^{3}H-thymidine uptake than the intact Ab (Fig 2). Similar Fc-mediated down regulation of the mitogenic properties of anti-Ig ligands has been previously observed with normal B cells.[37,38] Membrane IgM cross-linking was necessary for both forms of signaling in that Fab fragments of HB-57 were functionally compromised (data not shown).

Modulatory effects of activated T cell supernatant on signal transduction through membrane IgM in KON leukemic cells. Activated T cell supernatants and purified T cell factors, such as B cell growth factor (BCGF) and interleukin 2 (IL2) have been shown to exhibit synergy with soluble anti-IgM Ab in inducing the proliferation of normal resting B cells[18,39-44] and some malignant B cell populations.[6,42,43] The T cell factors are thought to promote the cell cycle progression of B cells that have received activation signals from membrane IgM cross-linking events. Our studies with KON leukemic cells, however, showed that the behavior of these cells in culture with activated T cell supernatants differed significantly from that of normal polyclonal populations of peripheral blood B cells (Table 2).

First, unlike normal B cell populations, KON cells showed significant increases in ^{3}H-thymidine uptake when cultured with T cell supernatant alone. The increases were manifest as early as two days of culture and peaked on day 4 of culture. T cell supernatants from three distinct lots tested all had maximal mitogenic effects at the highest concentration tested, ie, 25%, but could induce significant DNA synthesis at concentrations as low as 1% (data not shown). The KON cell activating moiety in the T cell supernatants is not due to residual PHA-P used to stimulate T cell factor production since, as shown in Table 3, a concentration of anti-PHA-P, which can completely abrogate the response of peripheral blood T cells to PHA-P, did not inhibit the leukemic cell response to T cell supernatant. Furthermore, KON cells showed no response to a concentration of PHA-P that would be maximally present in cultures containing 25% T cell supernatant.

A second and more surprising finding with KON cells, shown in Table 2, was that coculture with both anti-IgM Ab and T cell supernatant resulted in an antagonism in the induction of DNA synthesis, ie, the ^{3}H-thymidine uptake was significantly below that observed with either activator alone. The suppressed DNA synthesis noted on d4 of culture (Table 2) did not reflect a shift in the kinetics of peak ^{3}H-thymidine uptake in that significant inhibition was noted from d2-d8 of culture (data not shown). In contrast to KON cells, LUB cells did not respond to culture alone with T cell supernatant by further increases in ^{3}H-thymidine uptake nor

Table 2. Effect of Activated T Cell Supernatant on DNA Synthesis of Normal and Leukemic B Cells in the Presence and Absence of Anti-IgM Ab

Cells	100 µg/mL HB-57 Anti-IgM	cpm ^{3}H-Thymidine Uptake ($\bar{X}$ ± SEM)	
		No T Cell Supnt	+25% T Cell Supnt
Normal ER-peripheral blood cells	−	1,680 ± 57	1,961 ± 256
	+	4,343 ± 210	11,615 ± 883
KON leukemic B cells	−	145 ± 32	14,586 ± 948
	+	24,311 ± 898	3,855 ± 941
LUB leukemic B cells	−	32,643 ± 3,574	29,438 ± 1,045
	+	1,688 ± 176	2,209 ± 180

Normal human peripheral blood B cell preparation (ER negative cells) and KON and LUB leukemic B cells (2 × 10^5 per culture) were tested for responsiveness to soluble anti-IgM Ab in the presence and absence of 25% supernatant from activated T cell cultures. The data were obtained from three to five replicate cultures harvested on d4.

Abbreviation: Supnt, supernatant.

Table 3. Mitogenic Activity of T Cell Supernatant for KON Leukemic B Cells Is Not Due to Contaminating PHA-P*

Cells	Stimulant	cpm ^{3}H-Thymidine uptake ($\bar{X}$ ± SEM) No Ab	+ Anti–PHA-P (50 μg/mL)
KON	None	177 ± 39	197 ± 42
	T cell supnt (25%)	4,267 ± 157	4,041 ± 141
	PHA-P (0.5 μg/mL)	299 ± 64	252 ± 32
PB T cells (ER+)	None	560 ± 216	295 ± 79
	T cell supnt (25%)	556 ± 139	445 ± 44
	PHA-P (0.5 μg/mL)	13,720 ± 781	246 ± 65
	PWM (1:400 vol/vol)	18,242 ± 1,543	17,247 ± 3,697

*KON leukemic PB cells or ER positive cells from normal PB were cultured at 2 × 10^5 per well with the various activators in the presence or absence of 50 μg/mL purified anti–PHA-P Ab. The concentration of PHA-P mitogen tested was that which would be maximally expected to be present in cultures containing 25% activated T cell supernatant. KON cells were harvested on d4, normal T cells were harvested on d3 following an 18-hour pulse with 1 μCi ^{3}H-thymidine.

did the addition of T cell supernatant modulate the inhibitory effect of anti-IgM Ab on the background DNA synthesis of these cells.

The data shown in Table 2 suggest that, after exposure to factor(s) from activated T cells, KON cells become receptive to inhibitory rather than stimulatory signals following membrane IgM cross-linking. Further analysis of this phenomenon revealed that inhibitory signal transduction could be achieved by HB-57 anti-IgM MoAb concentrations that were orders of magnitude lower than those required for transduction of stimulatory signals in the absence of T cell supernatant (Table 4). Although the DNA synthesis in T cell supernatant-stimulated cultures containing high concentrations (200 μg/mL) of HB-57 anti-IgM was significantly less than that observed in cultures with either activator alone, significant levels of DNA synthesis were still detectable in these cultures, ie, 7,689 ± 258 cpm ^{3}H-thymidine uptake. This likely reflects a residual, although compromised, stimulatory signal from the high concentration of HB-57 anti-IgM Ab, which in the absence of T cell supernatant induced 72,789 ± 2,400 cpm ^{3}H-thymidine uptake. Supportive of this is the fact that anti-IgM MoAb XG9, which is not mitogenic for KON cells, causes >95% inhibition of T cell supernatant-induced DNA synthesis at ligand concentrations ranging from 0.5 to 200 μg/mL. It should be noted parenthetically that the difference in stimulatory signaling properties of HB-57 and XG9 MoAbs has also been observed with normal B lymphocytes[18] and reflects the distinct epitope specificity and cross-linking potential of these antibodies (S. Rudich and P. Mongini, manuscript in preparation).

Table 4. Inhibitory Signal Transduction in T Cell Supernatant Stimulated KON Leukemic B Cells Is Achieved by Low as Well as High Concentrations of Anti-IgM Ligands

MoAb	Conc (μg/mL)	cpm ^{3}H-Thymidine Uptake ($\bar{X}$ ± SEM) No T Cell Supnt	+25% T Cell Supnt
None	—	155 ± 19	16,927 ± 690
HB57 anti-IgM	200	72,789 ± 2,400	7,689 ± 258
	50	12,462 ± 352	2,205 ± 132
	10	575 ± 82	753 ± 176
	0.5	375 ± 126	557 ± 5
	0.05	265 ± 88	2,908 ± 29
	0.005	188 ± 34	12,594 ± 912
XG9 anti-IgM	200	395 ± 26	627 ± 64
	50	319 ± 16	506 ± 57
	5	273 ± 59	549 ± 57
	0.5	108 ± 18	646 ± 45
	0.05	144 ± 22	2,438 ± 59
	0.005	102 ± 49	15,221 ± 395

2 × 10^5 KON leukemic B cells were incubated in the presence or absence of activated T cell supernatant with various concentrations of HB-57 or XG9 anti-IgM MoAbs. Cultures were terminated on day 5 of culture after an 18-hour pulse with ^{3}H-thymidine.

The anti-IgM-mediated inhibitory signal transduction observed in T cell supernatant-activated KON cells mimics that observed with LUB cells (Fig 2). Thus, inhibition in both cases was mediated by very low ligand concentrations, and in both cases F(ab)2 fragments of anti-IgM MoAb were as efficient at transducing inhibitory signals as intact Ig (data not shown with T cell supernatant-activated KON cells).

Anti-IgM Ab does not induce terminal differentiation of leukemic cells. The negative effect of anti-IgM Ab on T cell supernatant-induced KON DNA synthesis and spontaneous LUB cell DNA synthesis does not reflect an induced differentiation to immunoglobulin-secreting plasma cells. Thus, KON cells did not secrete IgM, IgG, or IgA1, above the threshold of detection of the radioimmunoassays described in Materials and Methods, when precultured either in the presence or absence of anti-IgM ligand and/or T cell supernatant. While LUB cells did secrete a substantial amount of IgM after preculture in medium without Ab (2,920 ng/mL), the preculture of these cells with 0.010 to 100 μg/mL HB-57 resulted in a significant reduction in subsequent IgM production (≤6 ng/mL). Comparable inhibition of Ig secretion from CLL lymphocytes by anti-idiotype Ab has been previously reported.[44] There was no indication that the inhibition of IgM secretion in LUB cells was accompanied by a shift in the isotype of Ab synthesized (data not shown).

Culture of KON cells with both anti-IgM MoAb and mitogenic phorbol ester does not result in inhibition of DNA synthesis. Anti-IgM Ab did not induce a negative signal for DNA synthesis when added to KON cell cultures with another independent activator of these leukemic cells, ie, 0.8 μmol/L PMA. Rather, the coculture of 1 μg/mL HB-57 anti-IgM Ab and 0.8 μmol/L PMA resulted in ^{3}H-thymidine uptake somewhat greater than that seen with either stimulus alone. Thus, the ^{3}H-thymidine incorporation above background in cultures containing anti-IgM MoAb alone, PMA alone, or the combination of anti-IgM MoAb and PMA was 730 ± 22; 17,981 ± 907; and 29,264 ± 767, respectively (background = 318 ± 49). Similar phenomena were observed when doses of PMA as low as 0.0008 μmol/L were tested (data not shown).

Comparison of cell surface antigens on KON and LUB HCL lymphocytes. Cell membrane antigen expression on the two HCL cell populations that, in the absence of T cell factors, displayed contrasting responses to anti-IgM ligands was evaluated by use of the FACS. The data shown in Table 5 indicate that both KON and LUB leukemic cell populations were positive for IgM, kappa. While the KON cell population was brighter than the LUB population, both HCL clones expressed substantially greater amounts of mIgM than all studied CLL clones (data not shown). A very low level of normal B cell contamination in the KON cell preparation was indicated by an incidence of lambda light chain positive cells of only 1.4% above the gate set for 5% positive cells with an isotype matched control MoAb, J606. By this same comparison, the LUB cell preparation contained 6.1% lambda-positive cells above background.

Both KON and LUB leukemic cells clearly expressed CD20 pan B cell molecules and HLA-DR molecules as indicated by strong positive staining with MoAb B1 and MoAb S157, respectively. In addition, they both showed positive expression of an activation-associated molecule recognized by 4F2 and expression of CD19 (B4) and CD5 (Leu1-91d4). The latter is a membrane molecule found on all T lymphocytes, a small subpopulation of normal B cells and most chronic lymphocytic leukemias.[7,22] Both cell populations were very weakly stained by B2 (CD21), a marker most clearly expressed on less mature B cells.[31] Neither KON or LUB cells showed significant staining with J5 (CD10) or PC-1, indicators of precursor B cells and plasma cells, respectively.[32,33]

The major surface marker differences between KON and LUB cells appeared to be in the expression of membrane IgD and the B cell subpopulation marker FMC7, which is found on 50% of normal peripheral blood B cells and on most HCL.[23,24] While KON cells were consistently positive for mIgD and FMC7, the expression of these molecules on LUB cells was so weak as to be questionable.

It should be noted that in multiple instances the fluorescence intensity of leukemic cells positively stained with the B cell-directed Abs overlapped with that of control populations and thus the percentage of cells gated as positive was less than 100%. Since the staining profiles of both LUB and KON cells with these Abs never indicated a bimodal distribution (data not shown), the percent positivity in these cases likely represents the proportion of cells bearing the antigen above a certain threshold level of detection.

The functional characteristics of KON and LUB leukemic cell populations are not due to contaminating T cells and monocytes. The absence of consistently measurable numbers of contaminating T cells and monocytes by FACS analysis was characteristic of both the KON and LUB cell preparations (Table 5). Nevertheless, to eliminate the possibility that a low level contamination with these cells might contribute to the functional characteristics of the leukemic

Table 5. FACS Analysis of Membrane Antigen Expression on KON and LUB Leukemic Cells

		KON Leukemic Cells		LUB Leukemic Cells	
Ab Tested	Specificity	Percent Positive*	ΔMFI†	Percent Positive*	ΔMFI†
HB57	IgM	91.9	92	84.2	75
CBDA-4E5	IgD	80.6	45	7.5	10
HB61	Kappa light chain	94.3	102	80.2	64
PMG3A-4C6	Lambda light chain	1.4	−3	6.1	9
MθP-9 (LeuM3)	Pan monocyte	2.4	−11	3.7	6
89bl (CD3)	Pan T cell	−2.3	−6	1.0	0
B1 (CD20)	Pan B cell	92.1	125	85.9	76
B2 (CD21)	Limited B cell	26.8	15	8.7	14
B4 (CD19)	Pan B cell	76.9	37	57.1	32
FMC7	Limited B cell	37.7	20	13.2	7
J5 (CD10)	CALLA	3.2	0	−0.6	−1
PC-1	Plasma cell	7.7	5	12.6	15
Leu 1‡ (CD5)	Pan T and limited B	88.3	48	57.5	37
4F2	Activation antigen	85.3	43	31.4	26
S157	HLA-DR	84.1	75	89.1	72
5E9	Transferrin receptor	0.0	3	2.7	6

*The percent positive values represent the percent of cells that showed staining above background with the fluorescence threshold (gate) set at 5% positivity with each respective isotype control Ab. The values shown are the mean from two experiments in which frozen aliquots of the same leukemic cell preparation were used for each replicate experiment.

†The ΔMFI (mean fluorescence intensity) values were determined by subtracting the mean channel fluorescence observed with isotype matched control MoAb from that observed with the test MoAb. The values shown are the means from two experiments in which the logarithmic amplification of the fluorescence signal was similar (60 channels per decade in first experiment; 71 channels per decade in the second experiment on a 255 channel scale).

‡Leu 1 MoAb was used in one experiment while 91d4 MoAb, which recognizes the same molecule as Leu 1 and gave identical profiles to Leu1, was used in the second experiment.

Table 6. Functional Responses of KON and LUB Leukemic B Cells Depleted of Contaminating T Cells and Monocytes

		^{3}H-Thymidine Uptake ($\bar{X}$ ± SEM) Pre-culture Treatment With				
Cells	D 0-4 Culture In	Medium	C	Pan T MoAb	Pan Monocyte MoAb	Pan T + Pan Monocyte MoAbs + C
KON	Medium	615 ± 84	509 ± 48	250 ± 22	149 ± 32	252 ± 73
	1 μg/mL anti-IgM	589 ± 100	1,037 ± 144	439 ± 14	283 ± 120	556 ± 42
	100 μg/mL anti-IgM	14,327 ± 1950	36,398 ± 2705	23,357 ± 314	20,912 ± 131	31,355 ± 1572
	25% T cell supnt	15,924 ± 772	21,324 ± 1108	17,208 ± 636	12,758 ± 4214	15,846 ± 399
	1 μg/mL anti-IgM + 25% T cell supnt	691 ± 26	1,787 ± 254	676 ± 74	712 ± 25	806 ± 36
LUB	Medium	34,456 ± 1386	46,826 ± 1419	55,545 ± 1640	45,738 ± 4820	40,327 ± 836
	1 μg/mL anti-IgM	298 ± 64	318 ± 84	420 ± 35	331 ± 46	217 ± 13

KON and LUB cells were depleted of possible contaminating T cells and monocytes by treatment with pan T cell MoAb, and pan monocyte MoAbs and complement as described in Materials and Methods. 2×10^5 treated cells were then cultured with medium, 1 or 100 μg/mL HB-57 anti-IgM MoAb, and/or 25% activated T cell supernatant for four days. DNA synthesis was assessed by uptake of ^{3}H-thymidine during the last 18 hours of culture.

cell population, we tested the functional reactivity of KON and LUB cells following treatment with monocyte-specific and T cell-specific cytotoxic Abs plus complement. For depletion, conditions were used that had been established to specifically deplete monocytes and T cells from nonleukemic peripheral blood (see Materials and Methods section). The data in Table 6 indicate that all the phenomena seen with the untreated cell populations remain intact in the cell populations treated with the specific MoAbs and C.

DISCUSSION

The data presented here document that certain clonal populations of HCL B lymphocytes can receive either stimulatory or inhibitory signals for DNA synthesis when cultured in the presence of soluble anti-IgM antibody-supplemented medium. Inherent susceptibility to inhibitory signal transduction was evidenced in a leukemic clone, LUB, which exhibited significant background DNA synthesis. In contrast, stimulatory signal transduction was detected in a distinct clone, KON, which showed no background DNA synthesis. These studies substantiate a growing set of observations that certain transformed B cell populations are extraordinarily susceptible to in vitro growth regulation by membrane Ig cross-linking ligands.[11-13,42,43,45-47] The particular finding that one HCL clone, KON, receives stimulatory signals for DNA synthesis from soluble anti-Ig ligands without the need for accessory T cell-derived factors is uncommon but is in agreement with the recent report of Baeker and Rothstein with a poorly differentiated lymphocytic lymphoma.[11] Since B cell malignancies likely represent clonal expansions of normal B cell subsets, these latter observations are of particular importance in that they strongly imply that ancillary T cell factors are not obligatory for the proliferation of all human B cells and that membrane IgM crosslinking can, at least in some instances, suffice as the activating signal.

It should be emphasized that the DNA synthesis observed in KON leukemic cell preparations is highly unlikely due to the proliferation of contaminating normal B lymphocytes. First, as just mentioned, the anti-IgM induced KON cell DNA synthesis was independent of accessory T cell factors while normal peripheral blood B cells required ancillary T cell factors for optimal stimulation by this ligand. Second, FACS analysis, as well as visual cell surface immunofluorescence analysis, showed KON cells to contain insignificant numbers of lambda light chain positive cells, ie, ≤2%. This implies a maximal normal B lymphocyte contamination of 4%. Third, when normal B cells are cultured at 2×10^4 cells per culture (the number expected if normal B cells constituted even 10% of the leukemic cell preparation) no ^{3}H-thymidine incorporation above background is observed (unpublished results).

A unique observation made in this study was that activated T cell supernatants could modulate the outcome of membrane IgM-mediated signal transduction in the KON leukemic B cell population such that negative rather than positive effects on DNA synthesis were observed. This phenomenon became initially apparent on noting that KON cells responded with DNA synthesis to activated T cell supernatant alone or to high concentrations of soluble anti-IgM antibody alone but that they exhibited significantly depressed levels of DNA synthesis when cultured with both activating moieties simultaneously. Although observed at all ligand concentrations, anti–IgM-mediated inhibitory signal transduction was most pronounced at very low Ab concentrations that were nonmitogenic in the absence of T cell supernatant. Interestingly, the phorbol ester induced DNA synthesis of KON cells was completely resistant to anti-IgM mediated inhibitory signal transduction. This resistance may reflect the recently described capacity of PMA to block anti-IgM mediated phosphatidyl inositol metabolism and cytoplasmic Ca^{2+} increases in murine B cells,[48,49] although other explanations are also possible.

The antagonistic phenomenon exhibited when anti-IgM Ab and T cell supernatant were combined in culture was not due to supra-optimal signaling via a common activation pathway since (1) non-mitogenic anti-IgM MoAbs were capable of inhibiting T cell supernatant-induced KON cell DNA synthesis, and since (2) suboptimally mitogenic concentrations of anti-IgM MoAb HB57 inhibited the DNA synthesis induced by suboptimally mitogenic concentrations of T cell supernatant (unpublished results). Furthermore, the T cell supernatant-induced change in the nature of mIgM-mediated signaling in KON cells did not reflect a T cell factor-induced modulation of mIgM expression since FACS analysis of KON cells incubated with T cell supernatant for 2, 6, 24, 36, or 44 hours revealed levels of mIgM not significantly different from control cells (unpublished results).

Although it has previously been suggested that negative regulation of DNA synthesis through mIgM cross-linking is a phenomenon intrinsic to immature B cells and that positive signaling of DNA synthesis through mIgM is a phenomenon representative of more mature B cell subpopulations,[14,39] our observations with KON cells indicate that certain B cells expressing a mature functional phenotype can also be negatively regulated in cell cycle turnover by membrane IgM cross-linking events. In the KON clone, susceptibility to inhibitory or stimulatory signal transduction appears to be determined by the presence or absence of a T cell derived factor(s) that can independently activate the B cells. In this context, the observations of Linton and Klinman that recently stimulated secondary B cells exhibit a period of tolerance susceptibility to membrane Ig-binding ligands[50,51] may be particularly relevant.

The negative signal transduction in T cell supernatant-activated KON cells appears to be analogous to the mIgM-mediated inhibitory signaling inherent to LUB leukemic cells. We have considered it possible that LUB cells secrete an autocrine growth factor comparable to the KON cell stimulatory growth factor in activated T cell supernatants and that the mechanism of inhibition exhibited in both cell types is identical. However, extensive experiments have revealed no evidence for synthesis of such a growth factor by LUB cells. Thus, unlike lines that secrete autocrine growth factors,[9,52-55] conditioned medium from LUB cells, as well as cell lysates, did not augment the growth of limiting concentrations of LUB cells and did not induce S phase entry of either KON cells or anti-IgM stimulated normal B lymphocytes (unpublished results). It is alternatively possible that LUB leukemic cells express certain oncogenic elements that maintain them in a state analogous to the activation state reached by KON cells after exposure to T cell factors. In this regard, the leukemic cells described here could provide interesting models for testing the hypothesis that oncogenes mimic the biochemical events associated with certain ligand-receptor interactions.[56-58]

Analysis of cell membrane antigen expression on peripheral blood isolated KON and LUB leukemic cells indicated clearly that these HCL cells are of the B cell lineage. Since normal B lymphocytes have been shown to decrease their IgD expression following activation,[59] the presence of significant mIgD on KON cells but little or no mIgD on LUB cells is consistent with LUB cells representing a slightly more activated state. It is of interest that neither cell population exhibited a true resting B cell phenotype in that both expressed substantial amounts of the activation-associated molecule, 4F2.[25] However, these leukemic lymphocytes may represent activated cells that have reverted to a resting "secondary" 4F2-positive state as recently described in normal T cells by Suomalainen.[60] Consistent with this is their lack of significant transferrin receptor expression.

Although we have not yet identified the precise cytokine(s) from activated human T cells responsible for both activating the cells and modulating membrane IgM-mediated signaling, these effects of unseparated factors from polyclonally activated T cells on an isolated HCL B cell clone are likely relevant to the regulation of leukemic growth in vivo. The findings imply that activated T cells may have major roles in expanding the in vivo growth of certain leukemic B cell clones and furthermore, that in vivo treatment with mIg-binding ligands may be particularly useful for negatively regulating the T cell factor-enhanced growth of some of these leukemias as well as the spontaneous growth of certain other leukemias.

Since not all mIgM-positive leukemic B cell clones exhibit such exquisite sensitivity to mIg-mediated signal transduction, it would appear that in vitro screening of the responsiveness of distinct leukemic cell populations to anti-Ig ligands, both in the presence and absence of T cell factors, might increase the likelihood of effective in vivo anti-idiotype immunotherapy.[61] Clearly the efficacy of these in vivo Ab treatments at diminishing tumor load is likely to be greatly influenced by the characteristic way in which a malignant B cell population responds to anti-Ig ligands.

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CONCISE REPORT

Histone H1° Is Synthesized by Human Lymphocytic Leukemia Cells But Not by Normal Lymphocytes

By C. Mannironi, V. Rossi, A. Biondi, P. Ubezio, G. Masera, T. Barbui, and M. D'Incalci

Using a two-dimensional gel electrophoresis system (sodium dodecyl sulphate/acetic acid-urea-hexadecyltrimethylammonium bromide) coupled with fluorography, we investigated the synthesis of H1 isoproteins in leukemic cells obtained from peripheral blood of eight children suffering from acute lymphoblastic leukemia (ALL) (four T-ALL, three common ALL, and one B-ALL) and in normal peripheral lymphocytes. H1° was synthesized in leukemic cells but not in normal lymphocytes. Inhibition of DNA synthesis with hydroxyurea and 1-β-D-arabinofuranosylcytosine induced an increase of relative synthesis of H1° in leukemic cells but did not induce any detectable synthesis of H1° in normal lymphocytes. H1° synthesis was also undetectable in peripheral lymphocytes of leukemic children in complete remission after chemotherapy and in lymphocytes of a homozygotic twin of a leukemic patient. H1° may be a marker of malignant transformation of lymphocytes and as such could be of use for early detection of relapse in patients with acute lymphocytic leukemia in apparent complete remission.

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HISTONE H1 plays a role in maintaining and modulating the higher order structure of chromatin.[1,2,3] It is composed of a family of isoprotein species differing in primary structure.[4] Lennox et al,[5] using a two-dimensional electrophoresis system, recently resolved at least five H1 isoproteins plus H1° in different mouse tissues. In agreement with these studies D'Incalci et al,[6] using a two-dimensional electrophoresis system coupled with fluorographic detection, determined the synthesis of H1 and H1° in human cells growing in culture or in fresh human lymphocytes.

The relative synthesis of H1° isoproteins differs in growing and quiescent cells. Of the five H1 subtypes, H1.1, H1.2, and H1.3 were synthesized in proliferating and quiescent Go or G1 arrested cells; H1.4 and H1.5 were synthesized only in cells that were replicating DNA. As reported by other authors, H1° was synthesized more in quiescent than in proliferating cells,[7] and its relative synthesis increased after inhibition of DNA synthesis.[8,9] Several studies have suggested a correlation between H1° expression and cell differentiation.[10-13] Variable but appreciable H1° synthesis was found in all cell types investigated so far, except lymphocytes. Neither in T, B nor in large granular human lymphocytes was there any detectable synthesis of H1°.[6] In addition, Lennox et al[5,14] reported the lack of H1° in lymphocytes from blood, spleen, or thymus of mice, rats, rabbits, pigs, and calves.

This study was designed to evaluate the relative synthesis of H1 isoprotein species in human leukemic cells and compare it with that in peripheral lymphocytes from normal donors or from leukemic patients in complete remission.

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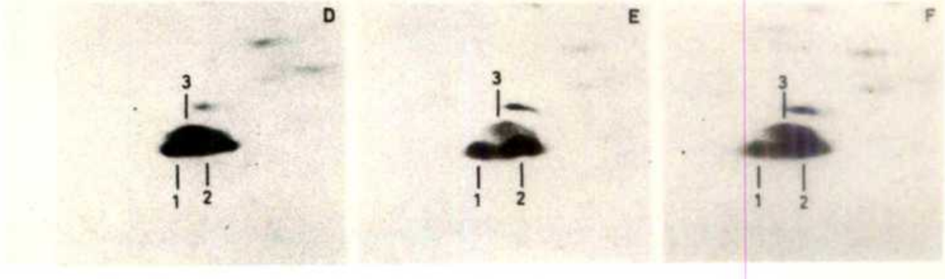

Fig 1. Synthesis of H1 isoproteins in human peripheral lymphocytes. H1 histone variants were extracted and analyzed on two-dimensional gels as described in Materials and Methods. (A) H1 isoprotein synthesis in quiescent lymphocytes. (B) Quiescent lymphocytes treated with 1 mmol/L HU and 10 μmol/L Ara-C. (C) PHA-activated lymphocytes. (D) Lymphocytes from peripheral blood of patient no. 5 in complete remission. (E) Lymphocytes of the homozygotic twin of patient no. 4. (F) Lymphocytes of the homozygotic twin, treated with 1 mmol/L HU and 10 μmol/L Ara-C. Percentage of cells in GO/1 = 95%, in S = 1.5%. Analysis was performed by flow cytometry.

MATERIALS AND METHODS

Cell culture. Mononuclear and leukemic cells were isolated from peripheral blood of normal donors and leukemic patients, respectively, by centrifugation on Ficoll Hypaque gradient. Cells were incubated overnight at 37°C in RPMI 1640 culture medium plus 10% fetal calf serum (FCS). Peripheral blood lymphocytes (PBL) and leukemic cells were stimulated to proliferate with 1 μg/mL phytohaemagglutinin (PHA) (Wellcome Research Limited, Beckenham, England) 48 hours before labeling.

Measurement of histone synthesis. To measure synthesis of H1 variants, PBL and leukemic cells were incubated for a labeling time of 2 hours at 37°C in lysine- and arginine-free RPMI 1640 medium supplemented with 10% FCS, 5 μCi/mL L-(U-^{14}C) lysine (348 μCi/mmol) and 10 μCi/mL L-(2, 3, 4, 5-^{3}H) arginine (52 Ci/mmol) (Amersham International, Bucks, England).

In experiments in which DNA synthesis was inhibited, 1 mmol/L hydroxyurea (HU) and 10 μmol/L 1-β-D-arabinofuranosylcytosine (Ara-C) were added 0.5 hours before labeling and again at labeling, and then left for 2 hours. HU and Ara-C were provided by the Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, MD.

Histone extraction. Whole cells were extracted with 0.5 N HCl containing 1% β-mercaptoethanol and 1 mmol/L phenylmethane sulphonyl fluoride (Sigma Chemical Company, St Louis) at 4°C overnight in a volume of 200 to 300 μL for 10^7 cells. After microcentrifugation (8,000 × g) for 5 minutes at 4°C, the supernatant was frozen and lyophilized.

Gel system. H1 isoproteins were separated by a two-dimensional polyacrylamide gel system previously described.[6,15] The first dimension consisted of sodium dodecyl sulphate (SDS) gel, the second of acetic acid/urea/hexadecyltrimethylammonium bromide (AUC).

Determination of radioactivity. Fluorograms were prepared by the method of Bonner and Laskey[16] and Laskey and Mills.[17] The gel was processed for fluorography with an autoradiography enhancer (Enhancer, NEN Research Products, Dreieich, W Germany). The dried gel was exposed to Kodak X-Omat AR films at −80°C for a period from 24 hours (for lymphocytes stimulated with PHA) to 2 months (for quiescent lymphocytes). A frame of radioactive ink (which comes out on the film) was drawn on the dried gel to ensure the precise placing of the gel on the film and accurate excision of the spots corresponding to H1 variants (five even when only three were visible on the gel) and H1°.

The excised spots from the gels were incubated overnight at 37°C in a 1 mL solution containing 95 parts 30% H_2O_2 and five parts concentrated NH_3 in a scintillation vial. Ten milliters acidified Aquassure scintillation liquid (NEN Research Products, Dreieich, W Germany) were added to each vial. Each sample was counted for ten minutes using a β-counter (Beckman LS 5800, Beckman Instruments, Irvine, CA). Pieces of gels that were negative at fluorography, randomly excised, were counted to determine background values, which were subtracted from the counts of each sample. Other spots visible on the film around H1 were analyzed, but their lysine/arginine ratios were clearly different from those for H1s, and therefore they are not additional H1 variants.

Flow cytometric analysis. DNA index and the proportion of cells in different phases of the cell cycle were determined by flow cytometry; DNA was stained with propidium iodide (PI) (Calbiochem, Behring Co, San Diego) by adding to the cell pellet 2 mL of PI solution, 50 μg/mL in 0.1% sodium citrate, 30 μL Nonidet-P40 detergent 1% (Sigma Chemical Co), and 15 μG RNAase (Calbiochem). Flow cytometry analysis was performed using a 30-L Cytofluorograf (Ortho Instruments, Westwood, MA). Percentages of cells in cell cycle phases were computed using the method of Baisch.[18] DNA index (DI) was computed by reference to human lymphocyte standards (DI = 1).

RESULTS

The typical pattern of H1 isoprotein synthesis in human normal lymphocytes is shown in Fig 1. H1.1, H1.2, and H1.3 are invariably synthesized in lymphocytes, but H1.4 and H1.5 only after a mitogenic stimulus (Fig 1C). Neither in PHA-activated nor in resting lymphocytes was H1° synthesized in detectable amounts.

The same pattern of H1 isoprotein synthesis was observed in lymphocytes isolated from the blood of leukemic children

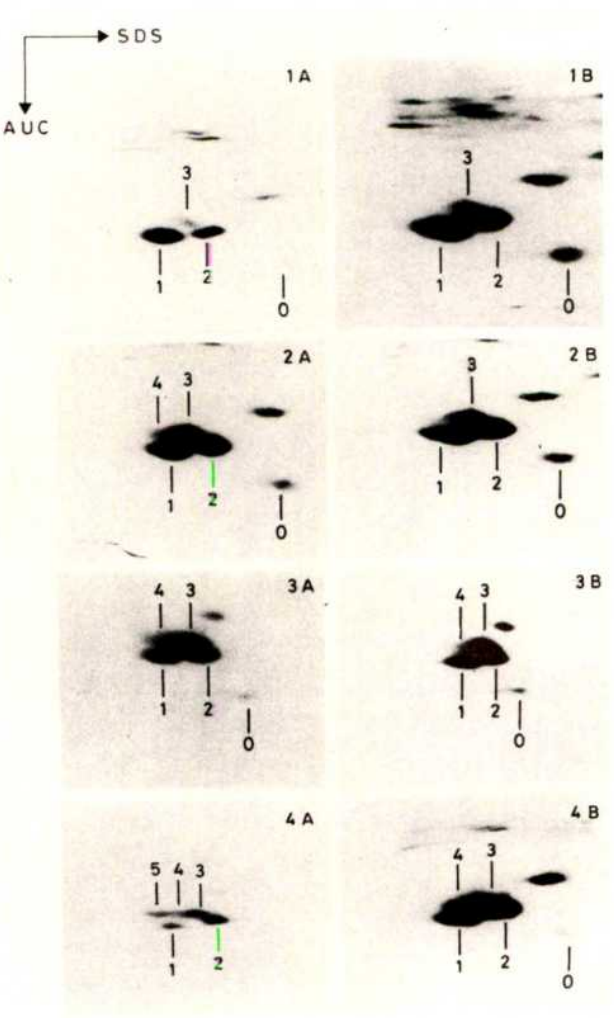

Fig 2. Synthesis pattern of H1 variants in leukemic cells of four T-ALL cases (nos. 1-4). (A) Untreated leukemic cells. (B) Leukemic cells treated with 1 mmol/L HU and 10 μmol/L Ara-C. Case no. 1: 17 years old; T-ALL; WBC 153,000/μL; blasts 98%; 95% cells in G0/1; 2% in S. Case no. 2: 13 years old; T-ALL relapsing after chemotherapy; WBC, 160,000/μL; blasts 99%; 88% of cells in G0/1, 9% in S. Case no. 3: 11 years old; T-ALL relapsing after chemotherapy; WBC, 110,000/μL; blasts 94%; 82% of cells in G0/1, 15% in S. Case no. 4: 3 years old; T-ALL; WBC, 135,00/μL; blasts 90%; 94% of cells in G0/1; 4% in S. All cases were males. DI was no. 1 in cases no. 1, 3, and 4 and hypodiploid (DI = 0.95) in case no. 2.

in complete remission after 3 months of chemotherapy (vincristine, 6-mercaptopurine, methotrexate, daunomycin) (Fig 1D) or in normal lymphocytes of a homozygotic twin of a patient suffering from acute lymphoblastic leukemia (ALL) (Fig 1-E, F). When quiescent lymphocytes were treated with DNA synthesis inhibitors, 1 mM HU plus 10 μM Ara-C, the synthesis pattern of H1 subtypes was not modified, and there was still no H1° synthesis (Fig 1B, F).

Figs 2 and 3 show that in leukemic cells from eight different cases of ALL, four T-ALL (Fig 2), three common ALL, and one B-ALL (Fig 3), H1.1, H1.2, and H1.3 were invariably synthesized (panels A). H1.4 and H1.5 were occasionally synthesized, presumably in relation to some DNA synthesis in these cells, since their synthesis disappeared completely (in the case of H1.5) or partially (in the case of H1.4) after HU and Ara-C treatment (panels B). In contrast to normal lymphocytes, H1° was synthesized in appreciable amounts in six out of eight cases investigated, and its synthesis increased proportionally after HU and Ara-C treatment, becoming evident in all cases. The H1° spot was also visible on the gel after Coomassie Blue staining (data not shown). Quantitative data on the synthesis of H1 subtypes and H1° in normal lymphocytes and in leukemic cells are shown in Fig 4.

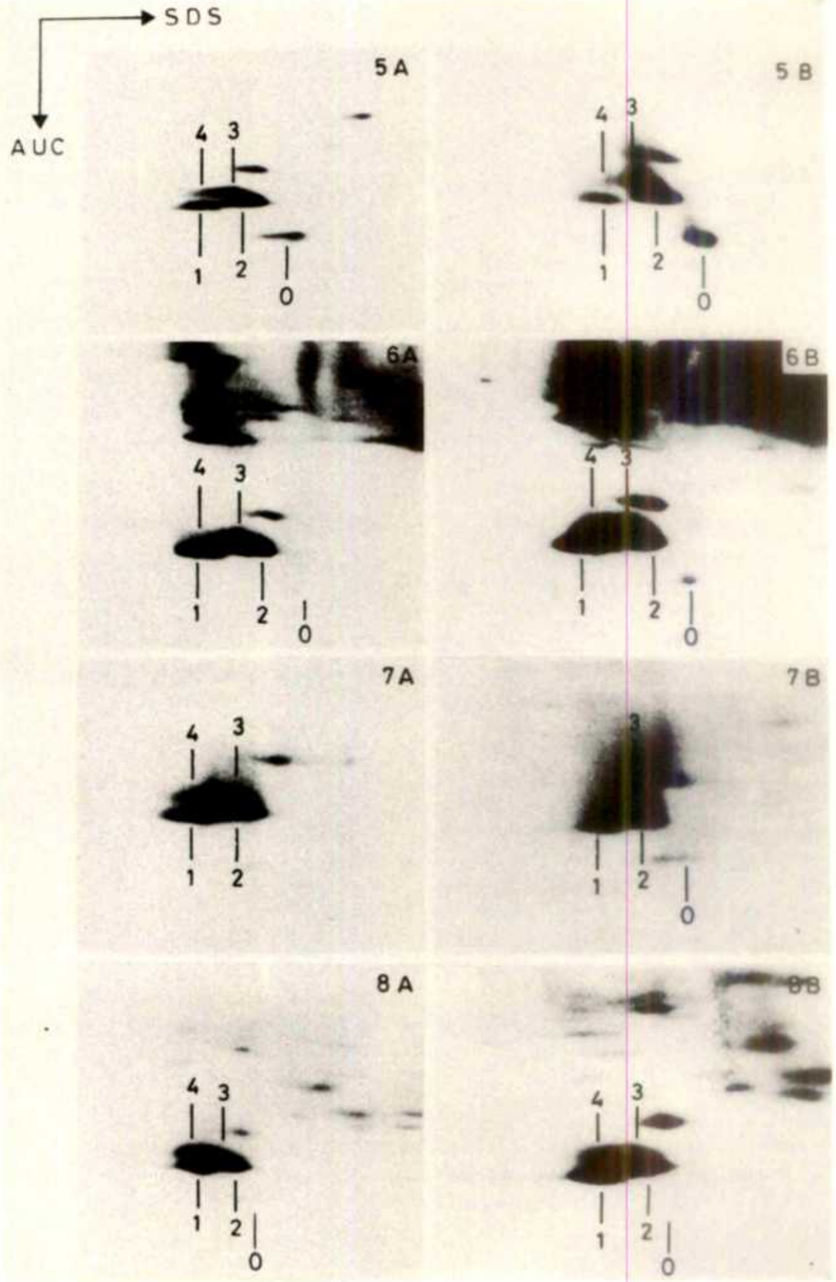

Fig 3. Synthesis pattern of H1 variants in leukemic cells of three common ALL (nos. 5, 6, and 7) and one B-ALL (no. 8). (A) Untreated leukemic cells. (B) Leukemic cells treated with 1 mmol/L HU and 10 μmol/L Ara-C. Case no. 5: 4 years old; common ALL; WBC, 126,000/μL; blasts 100%; 91% of cells in GO/1, 6% in S. Case no. 6: 1 year old; common ALL; WBC, 776,000/μL; blasts 100%; 93% of cells in GO/1; 4% in S. Case no. 7: 6 years old; common ALL; WBC 70,000/μL; blasts 90%; 95% of cells in GO/1, 4% in S. Case no. 8: 5 years old; B-ALL; WBC 41,000/μL; blasts 60%; 93% of cells in GO/1, 6% in S. All cases except case no. 6 were males; DI was no. 1 in all cases.

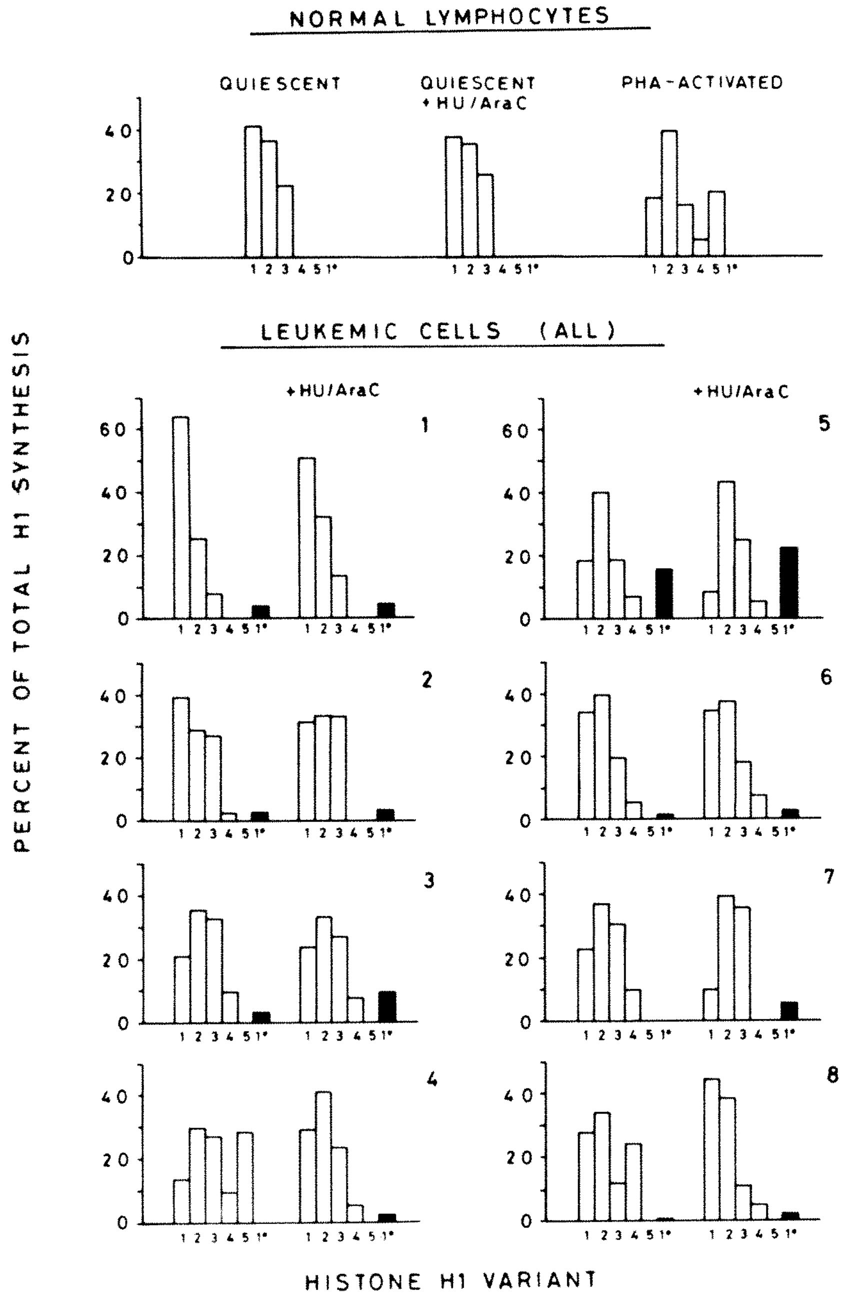

Fig 4. Quantitation of the synthesis of H1 isoproteins (white columns) and of H1° (black columns) in normal lymphocytes and in leukemic cells. Spots from gels shown in Figs. 1, 2, and 3 were excised and hydrolyzed. The amount of radioactivity in each spot was determined and expressed as a percentage of total H1 synthesis.

DISCUSSION

That normal human lymphocytes, in contrast to all other nondividing cells analyzed, do not synthesize H1° has already been reported.[5,6,14] Lennox et al,[5,14] who did not find detectable amounts of H1° in lymphocytes of peripheral blood, spleen, and thymus of various animal species, suggested that this exceptional chromatin feature might be related to the need of these cells to resume cell division during the immune response. The fact, however, that lymphocytic leukemic cells, which can also be stimulated to grow upon exposure with appropriate mitogens (eg, PHA or IL2 in T-ALL), synthesize H1° in detectable amounts contrasts with this hypothesis.

The finding that H1° is synthesized in leukemic lymphocytes but not in normal lymphocytes contrasts with the general idea that H1° is expressed more in better differentiated cells.

Indications exist that H1° plays a role in cell function regulation. Reportedly the synthesis of this protein is hormonally regulated in organs that are targets of hormonal control[13] (eg, thyroid, adrenal glands, and prostate). It has also been recently hypothesized that H1° plays a role in the regulation of gene expression.[19] An inactive gene, such as a-fetoprotein gene in adult mouse liver, was reported to be present in H1°-associated nucleosomes, whereas in the same cells an active unrepressed gene, such as the albumin gene, is not associated with H1°-containing nucleosomes. If H1° is a repressor of gene transcription, one can speculate that its synthesis in leukemic cells may be associated with the repression of genes that normally counteract the malignant transformation of lymphoid cells. The findings reported here, although referring to a very limited number of cases, suggests that H1° may be a marker of malignant transformation of lymphocytes and as such could be of potential clinical use for early detection of relapse in patients with acute lymphocytic leukemia in complete remission. Studies are in progress in our laboratory to obtain monoclonal antibodies against H1° to evaluate the presence of H1° *in a larger population* of patients suffering from lymphocytic leukemia.

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CONCISE REPORT

Geographic Differences in Hemophilia-Associated AIDS Incidence in Pennsylvania

By The Pennsylvania AIDS Surveillance Study Group

A 1986 survey of seven hemophilia treatment centers in Pennsylvania (PA) has revealed that 22 hemophiliacs residing in PA have developed the acquired immunodeficiency syndrome (AIDS), representing 9.2% of the total 238 United States hemophiliac AIDS cases. These 22 included ten (45.5%) from western PA (W-PA), eleven (50.0%) from central PA (C-PA), and one (0.5%) from eastern PA (E-PA). The HIV antibody prevalence for these three geographic groups is comparable, with 84 of 178 (47.2%) of hemophiliacs in W-PA seropositive, 102 of 182 (56.0%) in C-PA seropositive, and 105 of 177 (59.3%) in E-PA seropositive. Blood product usage for these three areas is comparable: 47.8 × 10^3 (W-PA) *v* 43.9 (C-PA) *v* 53.3 (E-PA) units factor VIII concentrate per patient per year; 36.5 *v* 24.5 *v* 33.7 for factor IX concentrate; 8.4 *v* 4.7 *v* 7.7 for cryoprecipitate; and 1.3 *v* 2.7 *v* 1.0 for fresh frozen plasma, respectively. These data demonstrate a geographic variation in hemophilia AIDS incidence in PA, with a tenfold higher incidence in W-PA and C-PA than E-PA, which is unrelated to differences in HIV antibody prevalence, patient blood product usage, or inaccuracies in AIDS case reporting. Because of the ≥5 year median latency between HIV infection and development of AIDS, the AIDS incidence will continue to change, but other factors appear to be operative in the development of AIDS in hemophiliacs.

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THE INCIDENCE of the acquired immunodeficiency syndrome (AIDS), identified in hemophiliacs early in the AIDS epidemic,[1] is quite high in hemophiliacs, with more than one case per 100 hemophiliacs.[2] Surveillance figures by the Center for Disease Control (CDC), however, have suggested some geographic variation in the distribution of United States hemophilia-associated AIDS cases, with some states reporting none or only a few cases and others reporting as many as eleven or more cases per 100 based on state hemophilia populations.[3] Presumably hemophiliacs were exposed to the human immunodeficiency virus (HIV), the etiologic agent, through infectious blood products, primarily factor concentrates, at about the same time as these products were manufactured and distributed nationally. This presumption is further corroborated by the documentation that the first HIV exposures in hemophiliacs occurred as early as 1978 and 1979 and the peak in seroconversion occurred in 1982 and 1983.[4-5] Thus, although the geographic variability in AIDS incidence remains unexplained, other factors could play a role, specifically differences in HIV antibody prevalence, yearly blood product usage, age, and accuracy of AIDS case reporting. Therefore, we studied these factors by distributing a questionnaire to the seven comprehensive care hemophilia treatment centers in Pennsylvania, and reviewed each center's state annual report for blood product usage and age.

MATERIALS AND METHODS

A questionnaire was distributed to the seven hemophilia treatment centers in Pennsylvania in September 1986, to determine the number of hemophilia AIDS cases in their center, the number tested for HIV antibody, the number HIV antibody positive, and the number and types of blood products used in that center. Only Pennsylvania residents at these centers were included. The centers included the Hemophilia Center of Western Pennsylvania (W-PA) (Pittsburgh) with 178 patients (178 tested), the Hemophilia Center of Central Pennsylvania (C-PA) (Hershey) with 182 patients (182 tested), and five centers in Eastern Pennsylvania (E-PA) with a total of 292 patients (177 tested). The latter included centers at Allentown Hospital (30 patients of which 15 were tested), Albert Einstein Medical Center (Philadelphia) (54 patients of which 27 were tested), Cardeza Foundation of Jefferson Medical College (Philadelphia) (90 patients of which 83 were tested), Children's Hospital (Philadelphia) (88 patients of which 32 were tested), and Reading Hospital (Reading) (30 patients of which 20 were tested). Information was obtained on a total of 537 hemophiliacs.

State annual reports for each hemophilia center for the years 1978 through 1985 were reviewed, and the mean age and mean number of units of each blood product used (factor VIII concentrate, factor IX concentrate, cryoprecipitate, and fresh frozen plasma) was determined. There was very little difference in age or blood product usage for a single center from year-to-year, and thus we used 1985 figures for the purposes of this report.

RESULTS

As shown in Table 1, HIV antibody prevalence in hemophiliacs from the three geographic areas of Pennsylvania was similar with between 47% and 59% of those tested HIV antibody positive. The proportion tested for HIV antibody in these centers included a total of 100% of the W-PA, 100% of the C-PA, and 61% of the E-PA hemophilia population. The seropositive included between 76% and 90% receiving factor VIII concentrate, between 31% and 52% receiving factor IX concentrate, between 0% and 11% of cryoprecipitate treated, and 0% (for all areas) receiving fresh frozen plasma. The 22 reported cases of hemophilia-associated AIDS in Pennsylvania included ten in W-PA, 11 in C-PA, and one in E-PA, representing 5.6%, 6.0%, and 0.3% of the hemophiliac population in these areas, respectively, and 11.9%, 10.8%, and 1.0% of the HIV antibody positive hemophiliacs in these three geographic areas, respectively.

Blood product usage, based on 1985 figures, in units × 10^3 per patient per year (U/p/yr) revealed 94 U/p/yr in W-PA, 76 U/p/yr in C-PA, and 96 U/p/yr in E-PA, with between 44 and 53 U/p/yr for factor VIII concentrate, between 25 and 37 U/p/yr for factor IX concentrate, between 5 and 8

From the Pennsylvania AIDS Surveillance Study Group. Participating members are listed in the Appendix

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Table 1. Geographic Variability in AIDS Incidence in Pennsylvania Hemophiliacs

Hemophilia centers	W-PA Pittsburgh	C-PA Hershey	E-PA Allentown Einstein, Philadelphia Cardeza, Philadelphia Children's, Philadelphia Reading	Total
No of AIDS cases (%)	10 (45 5)	11 (50.0)	1 (4.5)	22
HIV antibody prevalence (%)	84/178 (47.2)	102/182 (56.0)	105/177 (59.3)	291/537 (54 2)
AIDS incidence among HIV seropositive (%)	10/84 (11 9)	11/102 (10.8)	1/105 (1 0)	22/291 (7 6)
Blood product usage (U/pt/yr)*				
FVIII	47.8	43 9	53.3	
FIX	36.5	24.5	33.7	
CRYO	8 4	4.7	7.7	
FFP	1 3	2.7	1.0	
Age (yr)*				
<20	79 (37.3)	100 (50 8)	141 (40.8)	320 (42.4)
21–40	84 (39.6)	70 (35.5)	146 (42.2)	300 (39.7)
>40	49 (23.1)	27 (13.7)	59 (17.1)	135 (17 9)

*Taken from 1985 aggregate state hemophilia center annual reports.

U/p/yr for cryoprecipitate, and between 1 and 3 U/p/yr for fresh frozen plasma (Table 1).

The three geographic areas were not significantly different by age, based on 1985 figures with 42% of the hemophiliacs <20 years of age (range, 37% to 51%), 40% between 21 and 40 years of age (range, 36% to 42%), and 18% >40 years of age (range, 14% to 23%).

DISCUSSION

The results of this study demonstrate for the first time that geographic differences in hemophilia-associated AIDS incidence are unrelated to differences in HIV antibody prevalence. Specifically, the data from this survey of the seven Pennsylvania hemophilia treatment centers indicate that in Pennsylvania, where a total of 22 hemophilia-associated AIDS cases have been reported as of September 1986, there is a tenfold difference between the incidence of AIDS cases in the eastern part of the state and the central or western parts of the state, despite similarities in HIV antibody prevalence, blood product usage (per patient), or age distribution of hemophilia population in these geographic areas.

In view of the median 5 to 7 years incubation period between exposure and development of AIDS,[6] and with the peak years of seroconversion in hemophiliacs occurring in 1982 and 1983,[4-5] increasing numbers of AIDS cases will be seen over the next few years in areas of low AIDS incidence currently. However, it is puzzling that such wide discrepancies in AIDS incidence exist in areas of similar HIV seroprevalence. A recent collaborative CDC study by Jason et al and a large number of hemophilia treatment centers across the country[7] have shown that hemophiliacs exposed to "recalled" lots of factor concentrate (donor known to have subsequently developed AIDS) do not differ in HIV seroprevalence or T helper number from hemophiliacs exposed to nonrecalled lots of concentrate. These data would argue against a "bad lot" theory, that is, that some hemophiliacs or some hemophilia centers may have been exposed to a particularly infectious lot. Further, although a given hemophiliac may be exposed to a given lot of concentrate for several weeks to several months, and should this lot be a particularly "infectious" lot, there has been no evidence to support that he would be more likely to develop AIDS. On the contrary, review of lots of concentrate used by Pennsylvania hemophiliacs who subsequently developed AIDS has revealed no single common "bad" lot(s) exposure (Eyster ME, unpublished observation). Further, hemophilia brother pairs who received similar amounts of the same lots of concentrate over a several year period resulted in the development of AIDS or ARC in one but not the other brother of the pair (Ragni MV, unpublished observation). Whether the total dosage or frequency of exposure to a certain lot of concentrate plays a role in the development of AIDS is unknown.

Other possible explanations include the possibility that protective antibody or immunity to HIV may develop in some hemophiliacs rendering them "protected" from AIDS or that some hemophiliacs are exposed to less virulent strains of HIV and thus protected.

In conclusion, geographic differences in AIDS incidence in hemophiliacs in Pennsylvania appear to be unrelated to any geographic differences in HIV seroprevalence or blood product usage. These findings suggest that other factors are involved in the development of AIDS in hemophiliacs. Continued prospective surveillance of HIV infected hemophiliacs will be necessary to determine the risk factors associated with the development of AIDS in these individuals.

APPENDIX

The following organizations and members participated in this Pennsylvania AIDS Surveillance Study Group report. Hemophilia Center of Western Pennsylvania, Central Blood Bank and School of Public Health, University of Pittsburgh. Margaret V. Ragni, MD, Lawrence A. Kingsley, Dr P H , Joel A. Spero, MD, Jessica H Lewis, MD, Toni Gorenc; Hemophilia Treatment Center, Allentown

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CONCISE REPORT

Fructose-6-Phosphate,2-Kinase Activity in Human Erythrocytes

By Shinya Fujii, Masafumi Matsuda, Shigeru Okuya, Yoshiki Yoshizaki, Yukari Miura-Kora, and Toshio Kaneko

The hemolysate partially purified from human red cells was demonstrated to be capable of synthesizing fructose-2,6-bisphosphate (F-2,6-P$_2$) from fructose-6-phosphate in the presence of adenosine triphosphate (ATP) indicating that human red cells contain fructose-6-phosphate,2-kinase. The effect of F-2,6-P$_2$ on the rate-limiting enzymes of glycolysis, ie, hexokinase, phosphofructokinase (PFK), and pyruvate kinase, has also been examined. PFK was activated by this metabolite and the half-maximum activation was obtained at a concentration of 10^{-7} mol/L. Neither hexokinase nor pyruvate kinase was affected by F-2,6-P$_2$. These results suggest that human erythrocytes may contain this metabolite as one of the positive effectors for PFK.

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ERYTHROCYTE phosphofructokinase (PFK) is one of the major regulators of glycolysis. Adenosine triphosphate (ATP) serves as an inhibitor as well as a substrate. Inhibition by ATP reduces the affinity of the enzyme for its other substrate, frustose-6-phosphate (F-6-P). Considering physiological concentrations of ATP and F-6-P in erythrocytes, the enzyme functions at only 0.1% of its capacity, which corresponds to about 10% of the glycolytic rate.[1] Although this inhibition by ATP is mediated by ATP-Mg^{2+} complex, which is less inhibitory than ATP, the normal intracellular activity of PFK is largely dependent on the action of positive effectors, including adenosine monophosphate (AMP), adenosine diphosphate (ADP), fructose-1,6-bisphosphate (F-1,6-P$_2$) and glucose-1,6-bisphosphate (G-1,6-P$_2$).[2]

Fructose-2,6-bisphosphate (F-2,6-P$_2$) is present in the liver and is the most potent effector of hepatic PFK by releasing the ATP inhibition.[3] Although human erythrocyte PFK was also strongly activated by F-2,6-P$_2$, the absence of this metabolite in human erythrocytes has been reported.[4] The standard method to assay intracellular F-2,6-P$_2$ requires heating the tissue or cells at 80°C in an alkaline solution. Our preliminary experiment showed that it was extremely difficult to eliminate hemoglobin using this method, which, to a great extent, interfered with the F-2,6-P$_2$ assay system. In addition, chicken erythrocytes recently have been reported to contain this bisphosphorylated metabolite.[5] Therefore, these findings prompted us to reevaluate F-2,6-P$_2$ in human erythrocytes.

To test the possibility of its existence, we examined the enzyme's ability to synthesize F-2,6-P$_2$, fructose-6-phosphate,2-kinase (F-6-P,2-kinase) in the partially purified hemolysate and the effect of this metabolite on the rate-limiting enzymes.

MATERIALS AND METHODS

All reagents used were analytical grade. Commercial F-2,6-P$_2$ from Sigma (St Louis) was found to be contaminated with 0.1 to 0.4% F-1,6-P$_2$. Blood from a healthy volunteer was drawn into heparin and freed of white cells and platelets by passing it through a cellulose column.[6] After washing with 0.85% NaCl, the cells were lyzed in the 10 mmol/L phosphate buffer, pH 7.0. The stroma-free hemolysate was obtained by centrifugation and applied to DE 52 batchwise chromatography to remove hemoglobin.[7] The eluate was fractionated by adding ammonium sulfate. The fractions of 35% to 60% and 60% to 80% saturation were subjected to the following study after dialysis against 20 mmol/L HEPES bufer, pH 7.4.

F-6-P,2-kinase activity was measured as described elsewhere[8,9] by assaying F-2,6-P$_2$ synthesized from F-6-P in the presence of ATP The reaction mixture comprised 20 mmol/L HEPES, pH 7.4, 10 mmol/L MgCl$_2$, 1 mmol/L EDTA, 1 mmol/L dithiothreitol, 5 mmol/L ATP, various concentrations of F-6-P, and the enzyme After 20 minutes' incubation at 37°C the reaction was terminated by adding 100 μL of 0.5 N NaOH followed by heating at 80°C for 20 minutes. After cooling and centrifuging , half of the supernatant was neutralized with acetic acid in the presence of 20 mmol/L HEPES The formation of F-2,6-P$_2$ was determined by measuring its stimulatory effect on pyrophosphate-phosphofructokinase (PPi-PFK) in the presence of 0.5 mmol/L pyrophosphate and 1 mmol/L F-6-P[10] Since F-2,6-P$_2$ is easily destroyed under mild acid conditions, 0.5 N HCl was added to the other half of the supernatant. After standing at 25°C at a pH between 1 and 2 for ten minutes, the acid-treated supernatant was neutralized with NaOH and used as a control.

PFK and pyruvate kinase (PK) were assayed by the method of Beutler.[11] Hexokinase was assayed in the absence and presence of its effectors by the previous method.[7]

RESULTS

Figure 1 shows the formation of F-2,6-P$_2$ from F-6-P in the presence of the various fractions prepared from hemolysate by ammonium sulfate fractionation. In the fraction of 35% to 60% saturation, F-2,6-P$_2$ synthesis was seen, which was determined by the stimulation of PPi-PFK. In the control, stimulation was completely suppressed by preliminary treatment of the extract with HCl at pH 1 to 2. On the other hand, the fraction of 60% to 80% saturation showed no F-2,6-P$_2$ formation.

Figure 2 shows the effect of F-2,6-P$_2$ on PFK activity Km for F-6-P in the absence and presence of 1 μmol/L F-2,6-P$_2$ was 1.7 mmol/L and 0.7 mmol/L, respectively.

Figure 3 shows PFK activity as the function of F-2,6-P$_2$ concentration. With the concentrations of both 0.5 mmol/L (Fig 3A) and 1.0 mmol/L F-6-P (Fig 3B), ~90% maximum activation was obtained at 10^{-6} mol/L F-2,6-P$_2$ and the half-maximum activation was at 10^{-7} mol/L F-2,6-P$_2$

Table 1 shows the effect of F-2,6-P$_2$ on hexokinase activity in the absence and presence of various inhibitors. No effect was detected.

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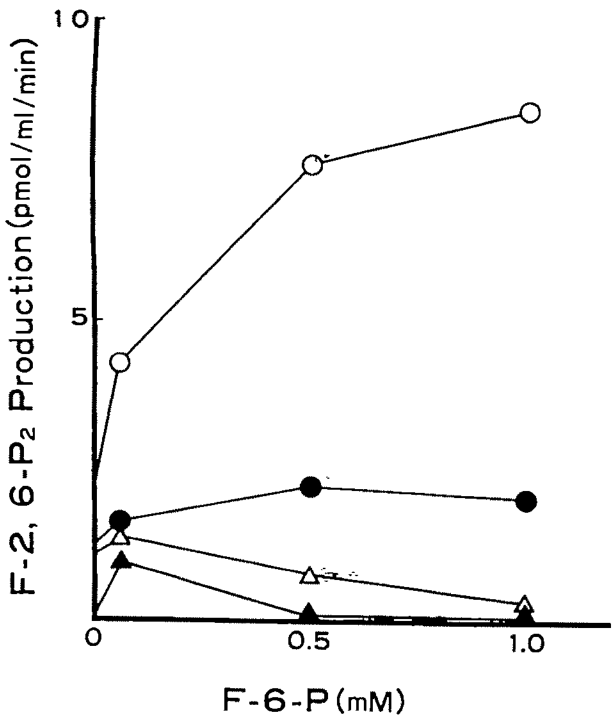

Fig 1. Formation of F-2,6-P_2 from F-6-P by the various fractions prepared from hemolysate by ammonium sulfate fractionation. Incubation procedure and assay system are described in detail in the text. (○—○) and (●—●), the fraction of 35% to 60% saturation; (△—△) and (▲—▲), the fraction of 60% to 80% saturation. After the incubation of each fraction with F-6-P and ATP, the reaction mixture was freed of proteins by boiling in an alkaline solution. After centrifugation, half of the supernatant as the control system (●—● and ▲—▲) was treated with HCl at pH 1 to 2 and then neutralized. The other half of the supernatant as the complete system (○—○ and △—△) was neutralized without preliminary acid-treatment. This is the representative of three separate experiments and each experiment was performed in duplicate.

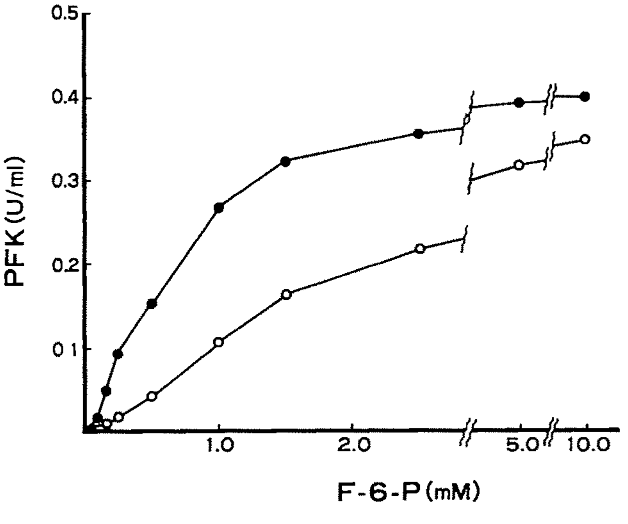

Fig 2. The effect of F-2,6-P_2 on PFK activity. In the absence (○—○) and presence (●—●) of 1 µmol/L F-2,6-P_2. Km for F-6-P was 1.7 mmol/L and 0.7 mmol/L, respectively. This is the representative of four separate experiments.

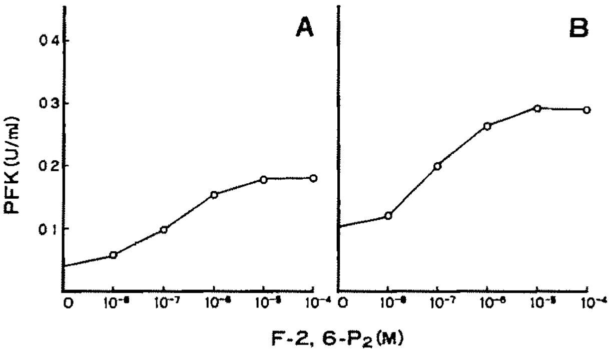

Fig 3. The effect of various concentrations of F-2,6-P_2 on PFK activity. Assay system for PFK activity contains 0.5 mmol/L F-6-P and 2.0 mmol/L ATP (A), and 1.0 mmol/L F-6-P and 2.0 mmol/L ATP (B).

Figure 4 shows the effect of F-2,6-P_2 on PK activity. The activity was stimulated in the presence of 1.0 mmol/L nontreated F-2,6-P_2. However, F-2,6-P_2 treated preliminarily with HCl at pH 1 to 2 also activated PK. In addition, similar activation was seen in the presence of F-1,6-P_2 at a concentration approximately the same as that contaminated in 1.0 mmol/L commercial F-2,6-P_2.

DISCUSSION

F-2,6-P_2 is acid-labile and alkaline-stable. The standard method for the determination of the glycolytic intermediates in red cells, which requires acid-treatment of the cells, is not used. On the other hand, alkaline-treatment at 80°C is not applicable either, because hemoglobin remaining after the treatment strongly interfered with the assay system for F-2,6-P_2 (data not given). Therefore, our present work has been to test the possibility of its existence, namely the demonstration of the enzyme activity to synthesize F-2,6-P_2 and the activation of PFK by this metabolite.

As shown in Fig 1, the fraction of 35% to 60% saturation was capable of synthesizing F-2,6-P_2 from F-6-P and ATP. The determination of this bisphosphorylated metabolite was based on its ability to stimulate PPi-PFK. Although it is not a direct determination, it is an established method. Well-known activators for PPi-PFK, such as ADP, AMP, F-6-P, F-1,6-P_2 and G-1,6-P_2, are all acid-stable, while F-2,6-P_2 is

Table 1. Effect of F-2,6-P_2 on HK Activity in the Presence of the Inhibitors

	F-2,6-P_2	
Additives	None	1.0 µmol/L
None	100	102.9 ± 4.2
G-6-P (25 µmol/L)	36.0 ± 1.1	38.3 ± 2.1
ADP (3.0 mmol/L)	65.0 ± 4.0	69.1 ± 0.9
G-1,6-P_2 (200 µmol/L)	80.7 ± 3 4	84.6 ± 2.8

Assay mixture for HK consisted of 50 mmol/L Tris-HCl, pH 8.0, containing 5 mmol/L $MgCl_2$, 0 2 mmol/L NADP, 1.0 mmol/L ATP, 0.1 U/mL G-6-PD and 50 µmol/L glucose (Km of HK for glucose) in the absence and presence of the physiological inhibitors. The mean values (±SD) were obtained from four to six independent experiments. No effects were observed even at 5 mmol/L glucose (data not given).

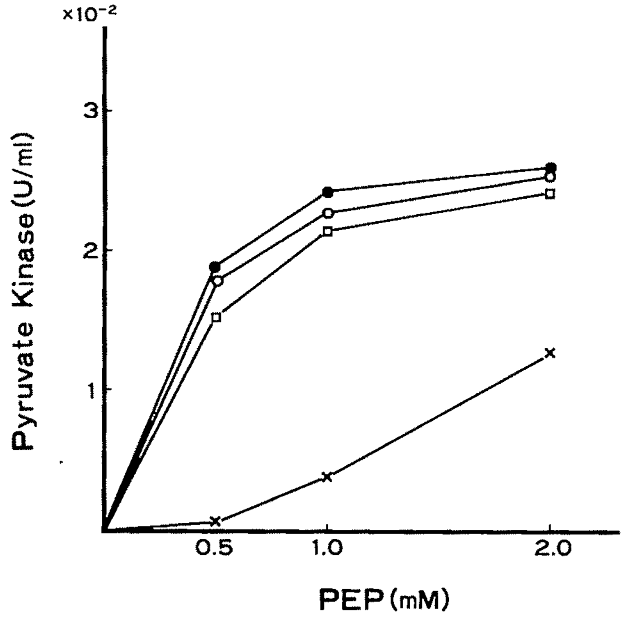

Fig 4. The effect of F-2,6-P_2 on PK activity. (X–X), Absence of F-2,6-P_2; (○——○), presence of 1.0 mmol/L nontreated F-2,6-P_2; (●——●), presence of 1.0 mmol/L acid-treated F-2,6-P_2; (□——□), presence of F-1,6-P_2 concentration which corresponds to that contaminated in 1.0 mmol/L F-2,6-P_2. PEP, phosphoenolpyruvate. This is the representative of eight separate experiments.

acid-labile and alkaline-stable. Up to now there have been no other compounds possessing these two properties.[10] In the control of the 35% to 60% saturation fractionation, F-2,6-P_2 was completely destroyed by the acid-treatment. These results indicate that human red cells contain F-6-P,2-kinase activity.

F-2,6-P_2 has been reported to be an effector of several enzymes in glycolysis, glycogenolysis, and gluconeogenesis.[3,12,13] However, erythrocytes lack the latter two pathways. Of the eleven enzymes in the glycolytic pathway, three appear to be particularly important: hexokinase, PFK, and PK. Hexokinase is the least active enzyme in the series and, therefore, often rate-limiting. Hexokinase is partially inhibited by some glycolytic intermediates and its activators act by releasing the inhibition.[2] As shown in Table 1, F-2,6-P_2 had no effect on the hexokinase activity even in the presence of these inhibitors such as G-6-P, G-1,6-P_2 and ADP.

PK from rat liver (L-type PK) has been reported to be activated by F-2,6-P_2.[14] Since erythrocyte PK is the same genetic product as L-type PK and has immunologically and kinetically similar properties, the activation of erythrocyte PK similar to that of L-type PK had been expected. However, as we show, erythrocyte PK was activated not only by nontreated commercial F-2,6-P_2, but also by acid-treated F-2,6-P_2, indicating that this activation was not due to authentic F-2,6-P_2. As commercial F-2,6-P_2 contains a minute amount of F-1,6-P_2, the activation of L-type PK as well as erythrocyte PK may be explained by the contaminant.

F-2,6-P_2 has been known to activate erythrocyte PFK.[4] However, the concentration for the half-maximum activation obtained here (10^{-7} mol/L) was lower than that of the previous report (10^{-6} mol/L)[4] and 10^{-6} mol/L F-2,6-P_2 was high enough to activate PFK up to 90% of its maximum effect (Fig 3). F-2,6-P_2 used here was also contaminated with F-1,6-P_2, but the concentration of the contaminant was too low to affect PFK activity.

From these results, it is strongly suggested that F-2,6-P_2 may be one of the intracellular constituents and may contribute to the regulation of PFK activity, and consequently, glycolysis in human erythrocytes.

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CONCISE REPORT

Platelet von Willebrand Factor: Evidence for Its Involvement in Platelet Adhesion to Collagen

By Edith Fressinaud, Dominique Baruch, Chantal Rothschild, Hans R. Baumgartner, and Dominique Meyer

Although it is well established that plasma von Willebrand Factor (vWF) is essential to platelet adhesion to subendothelium at high shear rates, the role of platelet vWF is less clear. We studied the respective role of both plasma and platelet vWF in mediating platelet adhesion to fibrillar collagen in a parallel-plate perfusion chamber. Reconstituted blood containing RBCs, various mixtures of labeled washed platelets and plasma from controls or five patients with severe von Willebrand disease (vWD), was perfused through the chamber for five minutes at a shear rate of 1,600 s^{-1}. Platelet-collagen interactions were estimated by counting the radioactivity in deposited platelets and by quantitative morphometry. When the perfusate consisted of normal platelets suspended in normal plasma, platelet deposition on the collagen was 24.7 ± 3.6 × $10^6/cm^2$ (mean ± SEM, n = 6). Significantly less deposition (16 ± 2.3) was observed when vWD platelets were substituted for normal platelets. In mixtures containing vWD plasma, significantly greater deposition (9 ± 2.2) was obtained with normal than with vWD platelets (1 ± 0.4) demonstrating a role for platelet vWF in mediating the deposition of platelets on collagen. Morphometric analysis confirmed these data. Our findings indicate that platelet, as well as plasma, vWF mediates platelet-collagen interactions at a high shear rate.

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VON WILLEBRAND FACTOR (vWF), a large multimeric glycoprotein, is synthesized by the endothelial cell that can release it into both plasma and the subendothelium.[1] It is also synthesized by the megakaryocyte, is present in the α-granules of platelets[2] and is released when platelets are stimulated by collagen, thrombin, and adenosine diphosphate (ADP).[2,3] Whereas plasma and subendothelial vWF clearly play a role in mediating platelet adhesion,[4-7] the role of platelet vWF is still unclear. Normalization of the bleeding time following 1-deamino-8-D-arginine vasopressin (DDAVP) infusion has been reported in type I von Willebrand disease (vWD) patients with normal but not with low platelet vWF.[8] In addition, in patients with type I vWD, the prolongation of the bleeding time correlates better with the vWF levels in platelets than in plasma.[8,9] The importance of the α-granule proteins released during platelet adhesion is further illustrated by studies of patients with storage pool deficiency.[10] In order to investigate the involvement of platelet vWF in mediating platelet adhesion to collagen we performed in vitro perfusion experiments using reconstituted blood from normal subjects and from patients with severe vWD.[11] Our results indicate that platelet vWF, in addition to plasma vWF, mediates both platelet adhesion and cohesion on fibrillar collagen.

MATERIALS AND METHODS

Materials. Equine tendon fibrillar collagen was from Hormon-Chemie, Munich, West Germany. Aggregation studies were performed in a Payton Dual-Channel aggregometer; ristocetin was from Lundbeck & Co, A/S, Copenhagen, Denmark. All chemicals purchased from commercial sources were of analytical grade (Fluka AG, Buch, Switzerland; Merck, France).

Patients. Five patients with severe vWD including two from the same family were tested. Studies to characterize these patients were as follows. Bleeding time was measured using a template device (Simplate II, General Diagnostics, Morris Plains, NJ). Factor VIII activity (VIII C) was assayed by a one-stage clotting technique[12]; vWF antigen (vWF Ag) by ELISA[13] and ristocetin cofactor activity of vWF (vWF RCo) using formalin-fixed platelets.[12] The concentration of these three parameters was expressed in international units (IU) per deciliter using as reference pooled normal plasma from 20 donors calibrated against the First International Reference Preparation for Factor VIII-related activities in plasma (National Institute for Biological Standards and Control, London). The platelet content of vWF Ag was measured by ELISA in washed platelets lysed by 0.2% Triton X-100.[9] The results of the above studies were essentially the same in all patients. Platelet counts and hematocrit values were in the normal range. The bleeding times were >30 minutes (normal range three to seven minutes), plasma levels of VIII C <5 UI/dL, vWF Ag $< 5 \times 10^{-2}$ U/dL in plasma (normal >50 U/dL), platelet vWF Ag values $<2 \times 10^{-5}$ U/10^9 platelets (normal >0.2 U/10^9 platelets). No patient had any inhibitor to vWF at the time of the study.

Preparation of perfusates. Venous blood from healthy volunteers or patients was collected in 1/10th volume of 0.108 mol/L trisodium citrate. Perfusates consisted either of whole citrated blood perfused immediately following collection or reconstituted blood. Platelets were isolated, labeled with chromium-51 (^{51}Cr), and washed three times as previously reported.[14] Several control studies were performed to verify that no significant amounts of plasma vWF remained adsorbed on platelets following washing: (1) washed platelets from control subjects did not aggregate in the presence of 1.3 mg/mL ristocetin unless a source of plasma vWF was added; (2) following platelet washing, the last supernatant contained $<5 \times 10^{-4}$U/mL of vWF Ag when measured by ELISA; and (3) by the same technique, no or only traces of vWF (5×10^{-3} U/10^9 platelets) were adsorbed on the platelet membrane.

Washed platelets were suspended in plasma and the final citrate concentration adjusted to 20 mmol/L. Perfusates were reconstituted

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immediately before perfusion by adding washed (three times) red cells to the suspended platelets (hematocrit 40%, platelet count 1.5×10^{11}/L). Four types of perfusates were used: (1) control platelets suspended in control autologous plasma; (2) vWD platelets mixed with control plasma; (3) control platelets mixed with vWD plasma; and (4) vWD platelets suspended in autologous vWD plasma. Mixtures contained blood components with similar blood groups.

Perfusion chamber and perfusion procedure. Perfusion studies were performed using a parallel-plate chamber, P1,[15] derived from the original device of Sakariassen et al.[16] Collagen (30 ug/cm^2) was sprayed onto plastic cover slips (Thermanox TM, Miles Laboratories Inc, Naperville, IL) and the collagen-coated surfaces were perfused at 37°C for five minutes at a shear rate of 1,600 s^{-1} with 20 mL blood samples as previously described.[14]

Quantitation of platelet-collagen interactions. Following perfusion, cover slips were cut parallel to the flow axis in two equal halves. One half was used for ^{51}Cr counting in a gamma counter (1260 multigamma II; LKB, Orsay, France). Platelet deposition was expressed as the number of deposited labeled platelets per cm^2.[17] The other half was used for morphometric evaluation. The percentage surface covered with platelets (platelet adhesion) and microthrombi higher than 2.5 μm (platelet thrombus formation) were estimated according to Baumgartner and Muggli.[18] Fixation, embedding and removal of exposed surfaces from cover slips were performed as previously described.[17]

Monoclonal antibodies to vWF. Two monoclonal antibodies (MoAbs) to human vWF were used. These MoAbs specifically inhibit vWF binding to its platelet membrane receptors: MoAb H9 inhibits its binding to glycoprotein Ib (GPIb)[19] and MoAb 9 blocks its binding to glycoprotein IIb/IIIa (GPIIb/IIIa).[19] In some experiments a MoAb to a mammary tumor glycoprotein (MTGP) was used as control.[20] IgG fractions were purified from ascites as previously described.[20] Perfusates were incubated for five minutes at 37°C with 20 μg/mL of MoAbs before perfusion.

Statistical analysis. Significance of grouped data was calculated with Student's *t* test for paired samples and *P* values <.05 were considered as significant.

RESULTS

Comparison of platelet adhesion to collagen in whole blood and in reconstituted blood. Perfusion experiments were first carried out to verify that washing the platelets did not modify their adhesion to collagen. Platelet adhesion to collagen was measured using either whole citrated blood or reconstituted blood from ten control subjects and three of the five patients with severe vWD. In controls, the percent surface coverage with platelets was similar in whole or reconstituted blood (Fig 1). As expected, platelet adhesion was markedly decreased in vWD as compared with the control group but no significant difference between whole or reconstituted blood was observed (Fig 1).

Effect of platelet and plasma vWF on platelet-collagen interactions using reconstituted blood from controls and patients with severe vWD. The perfusion experiments were performed with six control subjects and the five patients. One patient was tested on two occasions and identical results were obtained. The results of the four types of mixtures were compared for one patient and one control subject on each occasion under the same experimental conditions. When vWD platelets were suspended in normal plasma, platelet deposition was significantly less than when normal platelets were suspended in autologous plasma (Table 1) and this difference suggests that normal platelet vWF plays a role in platelet-collagen and platelet-platelet interactions. This was confirmed by results obtained with the last two mixtures: when normal platelets were suspended in vWD plasma, platelet deposition was significantly higher than when vWD platelets were resuspended in vWD plasma (Table 1). Results obtained by morphometric evaluation were similar to those using counting of radioactivity. Maximal platelet adhesion to collagen was obtained with mixtures of normal plasma and normal platelet vWF (Table 1). In mixtures containing control plasma the mean percent surface coverage with platelets (adhesion) was significantly less with vWD platelets than with control platelets. Further evidence that platelet vWF mediates platelet adhesion was obtained in studies using vWD plasma. In these studies the mean percent surface coverage with platelets was significantly higher with normal platelets than with vWD platelets (Table 1). The percent surface coverage with thrombi >2.5 μm showed parallel results to those of surface coverage with platelets in each of the tested mixtures (Table 1).

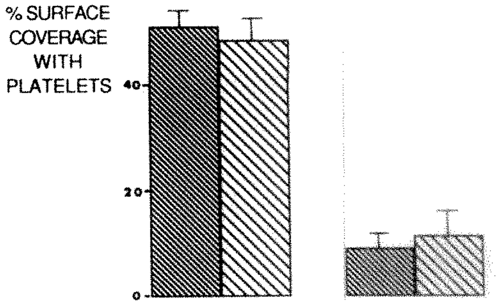

Fig 1. Comparison of platelet adhesion to collagen using whole blood or reconstituted blood from (left) controls (n = 10) or (right) patients with severe vWD (n = 3). Experimental conditions: equine collagen, 30 μg/cm^2; flow rate, 56 mL/min; shear rate, 1,600 s^{-1}; perfusion time, five minutes. Results represent mean ± SEM. ▩, whole blood; ▧, reconstituted blood.

Effect of monoclonal antibodies to vWF on platelet adhesion mediated by plasma and/or platelet vWF. The effect of MoAbs to vWF (H9 and 9) was tested in three perfusion experiments using mixtures containing normal

Table 1. Effect of Platelet and Plasma vWF on Platelet-Collagen Interactions in a Flow System Using Reconstituted Blood From Controls and Patients With Severe vWD

Perfusates		No. of Deposited Platelets (10^6/cm^2)	Percent Surface Coverage With	
Plasma	Platelets		Platelets	Thrombi >2.5 μm
Control	Control	24.7 ± 3.6	47.6 ± 4.5	38.4 ± 4.6
Control	vWD	16.0 ± 2.3*	41.0 ± 2.9*	29.4 ± 3.5*
vWD	Control	9.0 ± 2.2‡	24.5 ± 5.7†	14.6 ± 4.2†
vWD	vWD	1.0 ± 0.4‡	10.4 ± 1.4‡	2.3 ± 0.8‡

Experimental conditions same as in Fig 1. Results represent mean ± SEM of six experiments with six control subjects and five patients with severe vWD (one patient was studied twice). For each experiment, a control and a patient were simultaneously tested. *P* values compared with paired control perfusates (control plasma and control platelets).

**P* < .01.

†*P* < .0025.

‡*P* < .0005.

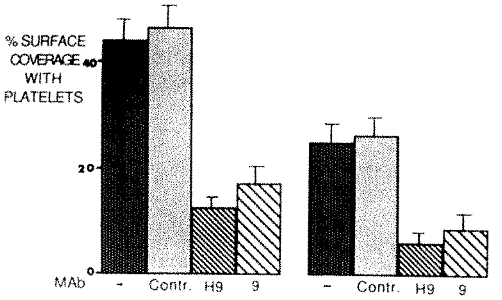

Fig 2. Effect of MoAbs to vWF on platelet adhesion mediated by plasma and/or platelet vWF. (left), normal plasma, normal platelets; (right), vWD plasma, normal platelets. Experimental conditions same as in Fig 1. Final concentration of MoAb IgG: 20μg/mL. Results represent mean ± SEM (n = 3).

platelets and either normal or vWD plasma. In the mixture containing normal blood components both MoAbs markedly inhibited platelet adhesion to collagen (Fig 2). This effect was specific as a control MoAb was without effect (Fig 2). In the mixture containing normal platelets and vWD plasma, both MoAbs also markedly inhibited platelet adhesion (Fig 2).

DISCUSSION

vWF mediates platelet adhesion to the subendothelium and to collagen at high wall shear rates.[4,6,14] Considerable progress in understanding the structure-function relationship of the vWF molecule in plasma has been achieved recently. At least two platelet membrane receptors, GPIb and GPIIb/IIIa, as well as three functional domains of vWF are required for optimal interactions between platelets, vWF and collagen.[21,22] The question whether platelet vWF can functionally substitute for plasma vWF in mediating adhesion of platelets to subendothelium is yet unanswered. Evidence for the hemostatic properties of platelet vWF is provided by clinical studies of patients lacking plasma but not platelet vWF.[8,9] Recently, Bowie et al[23] transplanted normal bone marrow into a pig with severe homozygous vWD and concluded that the platelet vWF alone partially corrected the abnormal hemostatic tests in severe porcine vWD. Our study on the respective influence of human plasma or platelet vWF on the interaction of blood with collagen-coated surfaces in a flow system is consistent with these studies. Under conditions of relatively high wall shear rates (1,600 s^{-1}) we confirmed that platelet adhesion to collagen is considerably impaired in vWD blood when both platelet and plasma vWF are absent. The defect in platelet adhesion was corrected to a considerable extent when normal plasma was substituted, but complete correction was not achieved. Similarly, with vWD plasma, both platelet deposition and surface coverage with platelets and with thrombi were greater with normal than with vWD platelets. Our results strongly suggest that platelet vWF plays a role in mediating platelet-collagen interactions at high shear rates. In these experimental conditions both platelet and plasma vWF are necessary for optimal platelet adhesion. In the absence of plasma vWF, platelet vWF can partially mediate platelet-collagen interactions.

We have previously shown,[15] as others have,[10,24] the involvement of both GPIb and GPIIb/IIIa in platelet adhesion to collagen and subendothelium mediated by vWF. In our experimental conditions, this function was blocked by two MoAbs to vWF, H9 and 9, directed against the vWF binding domain to GPIb and to GPIIb/IIIa, respectively. In the present study we tested the effect of these MoAbs to vWF on platelet adhesion suppressed interactions mediated by platelet vWF in the absence or in the presence of plasma vWF. Using vWD plasma, that is when vWF was only provided by control platelets, the inhibition of the percent surface coverage with platelets by either MoAb is an indication that platelet vWF acts in mediating platelet adhesion to collagen through the binding to the two same glycoproteins that are required for plasma vWF mediated platelet-collagen and platelet-platelet interactions.

In conclusion, platelet vWF is involved in vitro in platelet adhesion to collagen and probably also in vivo when platelet-released vWF accumulates in large quantities at the site of vessel wall injury.

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CONCISE REPORT

Secretion of Interleukin-1 by Acute Myeloblastic Leukemia Cells In Vitro Induces Endothelial Cells to Secrete Colony Stimulating Factors

By James D. Griffin, Alessandro Rambaldi, Edo Vellenga, Diane C. Young, Diana Ostapovicz, and Stephen A. Cannistra

The interaction of acute myeloblastic leukemia (AML) cells with stromal cells was investigated by adding AML-conditioned media to cultures of human endothelial cells. This conditioned media contained factors that induced expression of both the granulocyte macrophage colony-stimulating factor (GM-CSF) and granulocyte CSF (G-CSF) genes and release of colony stimulating activity from endothelial cells. The conditioned media contained interleukin-1 (IL-1) bioactivity and the endothelial cell stimulatory activity was partially neutralized by anti-IL-1 antiserum. Constitutive expression of the IL-1-beta gene was detected in ten of 17 AML cases analyzed. These results suggest that the unregulated secretion of IL-1 by AML cells can induce stromal cells in vitro to overproduce CSFs. This could contribute to the unrestricted growth of AML cells.

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ACUTE MYELOBLASTIC LEUKEMIA (AML) is a highly lethal hematopoietic malignancy characterized by excessive proliferation and aberrent differentiation of myeloblasts. A fraction of leukemic cells from most AML patients will also proliferate in vitro in semi-solid medium to form colonies of blasts.[1] In most cases, this requires the addition of colony-stimulating factors (CSFs), and recent studies with recombinant human CSFs have demonstrated that AML cells will proliferate in response to granulocyte CSF (G-CSF), granulocyte macrophage CSF (GM-CSF), and interleukin-3 (IL-3).[2,3] Thus, unlike most human myeloid leukemia cell lines such as HL-60, primary human leukemic cells remain factor dependent at least in vitro and presumably in vivo as well. The cells that supply hematopoietic growth factors in the marrow microenvironment are incompletely understood, but are likely to include endothelial cells, fibroblasts, macrophages, and T lymphocytes. While it is possible that the marrow microenvironment would normally supply adequate amounts of growth factors for a rapidly expanding leukemic clone, it is also possible that leukemic cells have developed mechanisms to augment their sources of growth factors. In this study, we demonstrate that AML cells constitutively secrete factors in vitro that induce stromal cells (endothelial cells) to secrete CSFs and that one of these factors is IL-1.

MATERIALS AND METHODS

Leukemic cells. Pretreatment bone marrow mononuclear cells cryopreserved in DMSO were thawed in the presence of human serum, depleted of T cells by rosetting with sheep erythrocytes, and depleted of monocytes by plastic adherence at 37°C for one hour. All specimens contained >95% blasts. Conditioned media was prepared by incubating leukemic cells at a concentration of 1×10^6 cells/mL in RPMI 1640 (Gibco, Grand Island, NY) with 10% fetal bovine serum (FBS) for 48 hours.

CSF assay. CSF activity was measured using a standard double layer agar assay for granulocyte-macrophage colony-forming units (CFU-GM) in which 5×10^4 light density, nonadherent, T depleted human marrow cells were cultured in a 0.3% agar overlayer and sources of CSFs or AML-conditioned media (AML-CM) (final concentration 10%) were in a 0.5% agar underlayer.[2] GCT medium (Gibco) was used as a positive control. Normal marrow cells were obtained from volunteers who gave informed consent approved by the institutional review board. CFU-GM in duplicate or triplicate cultures were counted on day 7.

Endothelial cells. Human umbilical vein endothelial cells (a gift of Dr Douglas Faller, DFCI) were grown in medium 199 (Gibco) containing 20% FBS, heparin 2 U/mL, and endothelial cell growth factor 25 μg/mL (Sigma, St Louis). AML-CM or control media was added to endothelial cells in a final concentration of 20% (vol/vol) and incubated for an additional nine hours before RNA extraction or 24 hours for CSF assay. In some experiments, purified anti-IL-1 antibody (Endogen Inc, Boston) was added to conditioned media (10 neutralizing U/mL) for one hour at 25°C before incubation with endothelial cells.

IL-1 assay. IL-1 activity of CM was assessed by measuring incorporation of tritiated thymidine by the IL-1 dependent AKR/J T cell clone D10.G4.1 (a gift of Dr Abul Abass, Harvard Medical School, Boston) in triplicate assays.[4,5] AML-CM was added to 2×10^4 D10.G4.1 cells in 150 μL containing 2 μg/mL concanavalin A (Con A) (Sigma). Recombinant IL-1-beta (Genzyme Corporation, Boston) was used to generate a standard curve. Duplicate wells without Con A served as a negative control. After 48 hours (37°C, 5% CO_2), the cells were pulsed with 1μCi ^{3}H-TdR (1.9 Ci/mmol/L, Schwartz-Mann, Spring Valley, NY) and harvested as previously described.[6] Data were expressed as IL-1 activity in units per milliliter from the standard curve.

Tumor necrosis factor assay. Tumor necrosis factor (TNF) activity was assessed by measuring cytotoxicity for WEHI-164 fibrosarcoma cells (a gift of Alberto Mantovani, Mario Negri Institute, Milan, Italy).[7] CM was added to 2×10^4 WEHI-164 cells in 100 μL medium containing 4 μg/mL actinomycin D (Sigma). Recombinant TNF (Asahi Chemical Co, Shizuoka, Japan) was used to generate a standard curve. For some experiments purified anti-TNF monoclonal antibody (Asahi) was added (10 μg/mL) for one hour at 25°C before the addition of WEHI-164 cells. After 18 hours (37°C, 5% CO_2), 10 μL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, 5 mg/mL in PBS, Sigma) was added, followed in four hours by addition of acid-isopropanol (100

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μL of 0.04NHCl in isopropanol). Absorbance was measured on a Titertek Multiscan Elisa plate reader (Flow Laboratories, McLean, VA) with a test wavelength of 570 nm. In this assay, 0.5 × 10^{-3}ng rTNF/mL was lethal to 50% of cells.

Northern blots. Four to 50 × 10^6 cells were lysed in 4 mol/L guanidium isothiocyanate and RNA was extracted, electrophoresed in agarose gels, and blotted as previously described.[8] Membranes were hybridized with ^{32}P-labeled probes as previously described. cDNA probes were labeled with ^{32}P-dCTP by the method of Feinberg and Vogelstein.[9] IL-1–beta, GM-CSF, and G-CSF mRNA were detected with cDNA probes provided by Drs Steven Clark and Gordon Wong, Genetics Institute, Cambridge, MA.[10] RNA from AML cells was derived directly from cryopreserved cells for some experiments, and from thawed, adherent-cell depleted, cultured AML cells in others.

RESULTS

AML-CM contains factors that induce CSF release by endothelial cells. Medium conditioned by purified blasts from four patients with AML was added to 1-mL cultures of human umbilical vein endothelial cells (EC) (10^5 cells) at a final concentration of 20%. The EC were then cultured for 24 hours and the medium removed for CSF assay. Control medium or medium from unstimulated EC did not contain detectable CSF activity in a standard CFU-GM assay (Table 1). The addition of AML-CM to EC cultures resulted in the production of readily detectable CSF activity for three of the AML cases (cases no. 1, 3, and 4). AML-CM from case no. 1 contained baseline CSF, in agreement with our previous observations,[8] but the amount of CSF present was enhanced after addition of this AML-CM to endothelial cells.

Culture of endothelial cells with AML-CM results in the induction of the GM-CSF and G-CSF genes. Northern blot analysis was used to investigate expression of the GM-CSF and G-CSF genes in EC. Cultures containing ~4 × 10^6 EC were incubated for nine hours with control medium or AML-CM, RNA was extracted and analyzed for the presence of GM-CSF and G-CSF transcripts (Fig 1). Unstimulated endothelial cells did not express either CSF gene. However, addition of CM from AML patient no. 4 resulted in the induction of expression of both CSF genes.

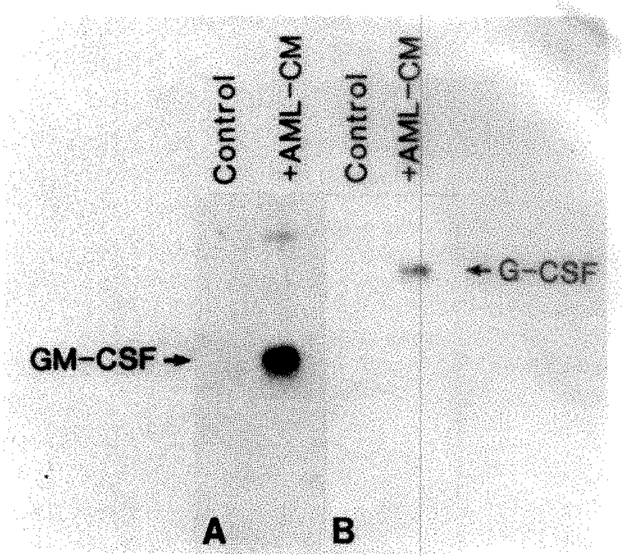

Fig 1. Northern blot analysis of EC stimulated by AML-CM. Total cellular RNA was extracted from 4 × 10^6 EC cultured with medium alone (control) or medium conditioned by AML blasts from case no. 4. One blot was hybridized successively with a cDNA probe for GM-CSF (left panel), washed, and rehybridized to a cDNA probe for G-CSF (right panel).

Table 1. AML-CM Induces Endothelial Cells to Release CSFs

Source of CSFs*	Day 7 CFU-GM/5 × 10^4 NBM cells
Medium alone	1 ± 0
AML-1 medium	39 ± 5
AML-2 medium	0 ± 0
AML-3 medium	4 ± 0.7
AML-4 medium	5 ± 4
EC medium	1 ± 0
EC + AML-1	99 ± 5
EC + AML-2	0 ± 0
EC + AML-3	62 ± 2
EC + AML-4	81 ± 7
GCT	43 ± 2

Abbreviations: NBM, normal bone marrow; GCT, conditioned medium from GCT cell line (positive CSF control).

*Control medium (RPMI 1640 with 10% FBS), media conditioned by AML cells or EC alone, or media conditioned by EC cultured for 24 hours with AML-CM were used at a final concentration of 10% in a CFU-GM assay.

The EC-stimulating activity present in AML-CM can be partly neutralized by anti–IL-1 antibodies. IL-1 and TNF have been previously shown to induce GM-CSF gene expression in EC.[11-14] We therefore measured levels of IL-1 and TNF in AML-CM using appropriate bioassays. CM from AML patients no. 1 to 4 contained 0.5, 0.075, >5, and >5 U/mL of IL-1 activity, respectively. TNF activity was <0.5 × 10^{-3} ng/mL in all four media. In order to test the possibility that one of the factors in AML-CM that induced EC release of CSF was IL-1, we incubated 200 μL aliquots of AML-CM with ten neutralizing units of anti–IL-1 antibody for one hour at room temperature, and then determined the residual ability of this medium to induce release of CSFs by EC. In the same experiments shown in Table 1, the addition of anti–IL-1 to CM from case no. 1 decreased subsequent CFU-GM formation from 99 ± 5 to 47 ± 9 CFU-GM/5 × 10^4 normal marrow cells. In case no. 4, anti–IL-1 reduced CFU-GM from 81 ± 7 to 17 ± 2. No effect of the antibody was observed in case no. 3 (62 ± 2 without antibody to 71 ± 2 with antibody), which also demonstrates the lack of toxicity of the anti–IL-1 antiserum. These results suggest that some, but perhaps not all, of the EC-stimulatory activity was due to IL-1.

Constitutive expression of the IL-1–beta gene in AML. Expression of the IL-1–beta gene in nonadherent AML blasts cultured for several hours in vitro was determined by Northern blot analysis in 17 cases of AML (Fig 2). RNA from the four AML cases described in Table 1 is shown in lanes 16, 14, 13, and 17, respectively. Expression of the IL-1–beta gene was observed in at least ten of 17 cases.

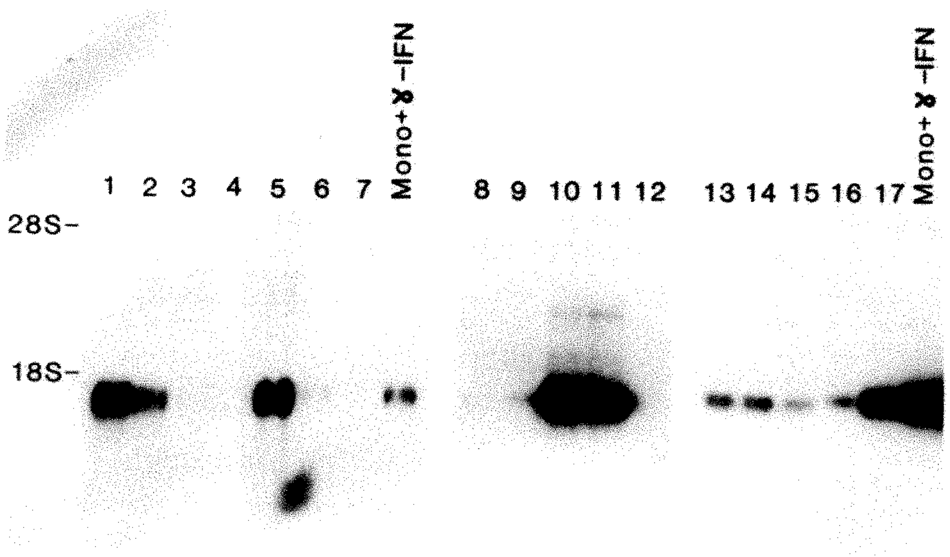

Fig 2. Northern blot analysis of IL-1–beta gene expression in primary AML cells. Total cellular RNA was extracted from 17 AML cases depleted of adherent cells and hybridized to a cDNA probe for IL-1–beta. Normal human blood monocytes isolated by plastic adherence were stimulated with gamma-interferon (100 U/mL) for 24 hours as a positive control.

There was no apparent correlation with the French-American-British (FAB) classification (four of four M1 cases were positive, as were two of three M2 cases, two of four M4 cases, and one of four M5 cases, one unclassified case was also positive). RNA was also extracted directly from cryopreserved AML cells from five patients in order to determine if the plastic adherence step induced IL-1 beta expression. Four of five cases had IL-1 beta mRNA (data not shown).

DISCUSSION

Growth of clonogenic AML cells in semi-solid media generally depends on the addition of CSFs. Recent studies from our laboratory have demonstrated that although expression of the genes for GM-CSF, G-CSF, and M-CSF in AML cells can be frequently detected, the amount of CSFs secreted is generally not sufficient to support in vitro leukemic colony growth by itself.[8] Also, many cases have been identified in which no CSF gene activation or secretion of CSF can be detected. We therefore considered the possibility that AML cells might secrete factors that could induce cells of the marrow microenvironment to release CSFs. AML-CM of four patients was tested for CSF activity and for its effects on CSF release by human umbilical vein EC. AML-CM of three of the four cases contained factors that induced expression of both the GM-CSF and G-CSF genes and release of CSF activity from EC. These results indicate that (1) there is a possible humoral interaction in vitro between AML cells and stromal cells that results in an enhanced supply of CSFs to leukemic cells, and (2) that these AML factors promote CSF production by enhancing gene expression rather than by inducing release of preformed CSFs by EC.

Reports from many laboratories have recently indicated that two monokines, IL-1 and TNF, can induce EC and fibroblasts to secrete CSFs.[11-14] We therefore assayed IL-1 and TNF levels in AML-CM. The results indicated that high levels of IL-1 activity could be detected in some, but not all samples, while the levels of TNF were below our detectable range. Further, some of the endothelial cell-inducing activity in AML-CM could be neutralized by a polyclonal antiserum to IL-1. These data suggest that one of the endothelial cell activating factors is likely to be IL-1. This was confirmed by Northern blot analysis, which demonstrated the presence of IL-1–beta mRNA in ten of 17 AML cases depleted of adherent cells and four of five cases in which there was no in vitro culture. IL-1 is known to be released by activated normal monocytes,[15] and production of IL-1 has also been observed by the monocytic "leukemic" cell lines U937[13] and THP-1.[16] Furukawa et al[17] recently showed that AML patients of the M4 or M5 subtype may secrete a factor that induces murine thymocyte proliferation and is presumably IL-1; Lachman et al[18] previously described the characterization of a "lymphocyte-activating factor" from acute myelocytic and myelomonocytic leukemia cells.

The results presented here suggest that IL-1–beta is one of the factors released by AML cells in vitro that can promote CSF release by EC. Expression of the IL-1 gene in AML cells is aberrent in the sense that it is "constitutive" and thus unregulated. Unregulated secretion of IL-1 by AML cells in vivo (possibly with other factors such as TNF) could result in unregulated production of CSFs by marrow stromal cells. We suggest that constitutive release of IL-1 by AML cells could contribute to the unrestricted growth of these cells, particularly in cases that do not produce their own CSFs.

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CONCISE REPORT

Acquisition of Formyl Peptide Receptors During Normal Human Myeloid Differentiation

By Richard Sullivan, James D. Griffin, and Harry L. Malech

By using a newly developed immune rosetting technique to isolate highly purified populations of myeloid precursor cells from normal human bone marrow and then inducing their differentiation with granulocyte and macrophage colony-stimulating factors (G/M-CSFs) in vitro, we studied the surface expression of chemotactic peptide receptors as the cells matured from the stage of the myeloblast to that of the mature, segmented neutrophil. We used ethylene glycol bis(succinimidyl succinate) to link *N*-formyl-Nle-Leu-Phe-Nle-[^{125}I]iodo-Tyr-Lys to chemotactic peptide receptors on the surface of myeloid cells at sequential stages of maturation and then determined the density of receptor-radioligand complexes by autoradiography after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Specific, saturable formyl peptide receptors were not detectable at the level of the myeloblast but gradually emerged through progressive stages of neutrophil maturation. The specific receptors for formyl peptide that appeared during cellular maturation had a mol wt of 55 to 70 kiloDalton (kD), corresponding to those present on the surface of peripheral blood neutrophils, and binding of the radioligand was highly specific in that it was completely inhibited by a 1,000-fold excess of F-Met Leu Phe. These data correlate with and provide insight into our recent observation that F-Met Leu Phe-induced membrane depolarization and transient increases in cytosolic free calcium are gradually acquired as neutrophils mature. This report represents to our knowledge the first description of the maturational development of chemotactic peptide receptor expression in normal human myeloid cells.

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APPROXIMATELY 10^5 receptors for formylated chemotactic peptides are present on the surface of the mature human neutrophil.[1] Occupancy of these receptors by *N*-formyl oligopeptides, which are byproducts of bacterial protein synthesis, sets into motion a coordinated array of cytotoxic and microbicidal activities by these cells that culminate in directed cell movement, degranulation, enhanced surface adhesiveness, and generation of the oxidative burst.[2] The transduction pathway that couples the occupancy of this receptor to the diverse cellular responses that it evokes is only partially defined but is currently the subject of intense investigation. A transient increase in free cytosolic calcium ions ($[Ca^{2+}]_i$)[3] and rapid depolarization of the plasma membrane[4] are prominent ionic events that immediately follow occupancy of chemotactic peptide receptors and may serve as important signals in the stimulus-response coupling of this receptor.

We recently developed a method to isolate[5] highly enriched populations of myeloid precursors from normal human marrow and to induce[6] their complete maturation to neutrophils in ten-day liquid cultures by the addition of granulocyte and macrophage colony stimulating factors (G/M-CSFs). By applying this technique, we found that F-Met Leu Phe-induced membrane depolarization and transient increases in $[Ca^{2+}]_i$ were completely absent in myeloblasts and gradually evolved as the cells matured.[6] In this report, we describe further studies that indicate that the incremental development of these ionic responses to F-Met Leu Phe during the granulocyte's ontogeny is paralleled by the gradual acquisition of specific receptors for formyl peptides.

MATERIALS AND METHODS

Isolation of myeloid precursor cells. Myeloid precursor cells from the stage of the committed G/M progenitor cell (CFU in culture, or CFUc) through the stage of the myeloblast were enriched ~100-fold using an immune rosetting technique that we previously described in detail.[5,6] Monoclonal antibodies used to deplete differentiated lymphoid and myeloid cells from marrow cell suspensions included α-MY8 and α-Mo1 (panmyeloid), α-MY4 (monocyte), α-B1 (B lymphocyte), α-T3 and α-T11 (T lymphocyte), 31C6 (NK cell and basophil, kindly provided by Drs J. Ritz and T. Hercend, Dana-Farber Cancer Institute, Boston) and α-glycophorin A (erythroid). Cells binding these antibodies were then eliminated on sodium metrizoate-Ficoll gradients after being allowed to form rosettes with sheep erythrocytes coated with rabbit anti-murine immunoglobulin.

Differentiation of myeloid precursor cells in liquid suspension cultures. One to 2×10^6 isolated myeloid cells were suspended in McCoy's 5A medium (Gibco, Grand Island, NY) containing 10% heat-inactivated fetal calf serum, penicillin (50 IU/mL), and streptomycin (50 μg/mL), at a concentration of 5×10^5 cells per milliliter. As a source of G/M-CSFs, 5% GCT medium (Gibco) and 5% medium conditioned by the Mo cell line (Gift of Dr Steven Clark, Genetics Institute, Cambridge, MA) were added, and the culture was incubated at 37°C in a humid incubator containing 5% CO_2 for ten days. At appropriate intervals, total and differential cell counts were performed.

Affinity labeling of chemotactic peptide receptors. Affinity labeling of formyl peptide receptors on whole cells was performed as previously described.[7] All labeling experiments were carried out

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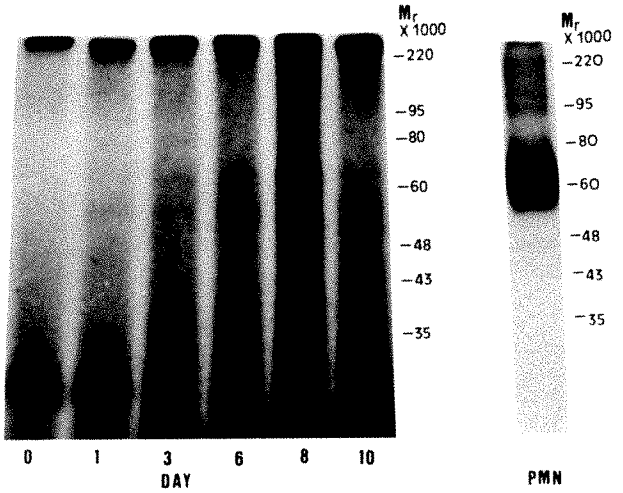

Fig 1. Autoradiograph of human myeloid cells fixed at sequential stages of maturation in culture, affinity labeled as described, and analyzed by SDS-PAGE (10^5 cells per lane). Day 0 indicates cells isolated directly from marrow and consists of myeloblasts and earlier forms. For comparison, peripheral blood granulocytes (10^6) were similarly analyzed.

either on whole fresh cells or on whole cells fixed with paraformaldehyde. For experiments represented by Fig 1, aliquots of cells were removed from the culture at intervals between zero and ten days, fixed in 1% paraformaldehyde, washed in phosphate-buffered normal saline, pH 7.4 (PBS), and then stored at 4°C until affinity labeling. For the experiments represented in Fig 2, cells were not fixed in paraformaldehyde and stored but instead were taken fresh from the cultures and affinity labeled immediately after washing. In brief, the hexapeptide analogue of F-Met Leu Phe, *N*-formyl-Nle-Leu-Phe-Nle-Tyr-Lys (Bachem, Inc, Torrence, CA) was labeled with ^{125}iodine (*N*-formyl-Nle-Leu-Phe-Nle-[^{125}I]iodo-Tyr-Lys). Affinity labeling of fixed or fresh whole cells was performed using 50 nmol/L *N*-formyl-Nle-Leu-Phe-Nle-[^{125}I]iodo-Tyr-Lys and ethylene glycol bis(succinimidyl succinate) (Pierce Chemical Co, Rockford, IL). Labeled cells were extracted with Triton X-100 in tris-glycine buffer and subjected to SDS-PAGE and autoradiography as previously described.[7] In a control study, we determined that paraformaldehyde fixation of granulocytes did not decrease affinity labeling of receptors, did not alter the molecular weight of receptor detected, and did not inhibit the ability of the detergent to solubilize the receptor. However, there was some decrease of receptor that could be affinity labeled after granulocytes were fixed and stored at 4°C for eight days, but no change in the apparent molecular weight of receptor labeled.

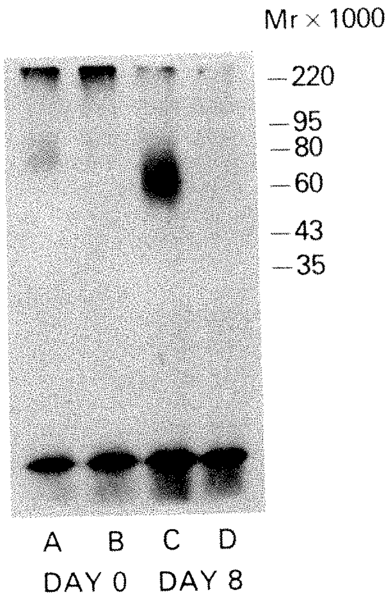

Fig 2. Autoradiograph of fresh, unfixed myeloid cells affinity labeled as described and analyzed by SDS-PAGE (10^6 cells per lane). Day 0 indicates cells isolated directly from marrow consisting of myeloblasts and earlier forms. Day 8 indicates cells allowed to mature for eight days in culture along the granulocyte pathway. Cells were affinity labeled in the presence (B and D) or absence (A and C) of a 1,000-fold excess of F-Met Leu Phe.

RESULTS

The total and differential cell counts obtained using the technique for myeloid cell isolation and *in vitro* differentiation that we employed in this report have been described previously in detail.[6] Immediately after the cells were isolated from the bone marrow, the vast majority (≈85%) were myeloblasts or morphologically unidentifiable, immature hemic precursors, as assessed by Wright-Giemsa staining. After 24 hours in culture, a substantial number of promyelocytes became evident (≈55%), with very few cells beyond that stage of development. Thereafter, a progressive wave of maturation took place, so that by day 8, most of the cells (≈75%) were postmitotic neutrophils, and by day 10, ≈65% were fully segmented neutrophils.

The acquisition of the formyl peptide receptor complex at sequential stages of cellular maturation in a representative experiment from a single donor is shown in Fig 1. Each lane shows the binding on successive days of iodinated hexapeptide to 10^5 cells that were removed from the culture, fixed with paraformaldehyde, and studied by covalent affinity labeling. On day 0, at which time very few cells more mature than the myeloblast were present, binding was undetectable under these conditions. On day 1, when the predominant type of cell in the culture was the promyelocyte, a single band appeared that had an apparent mol wt of ≈55,000. Labeling of this band could be completely inhibited by a 1,000-fold excess of F-Met Leu Phe (data not shown). By day 3, when considerable maturation past the promyelocyte stage had taken place, an additional band with a higher mol wt was visible that also represented specific saturable receptor. Thereafter, as the cells matured through the stage of the mature granulocyte, both bands became progressively more intense.

Since storage of fixed cells leads to some loss of detectable formyl peptide receptor, freshly isolated live day 0 myeloid precursors were affinity labeled and analyzed without fixation or storage. These were compared with similarly treated day 8 cultures derived from a different donor. Ten times more labeled cells (10^6) were loaded in each lane of the experiment shown in Fig 2 than were analyzed in each lane in Fig 1 in order to increase further the sensitivity of detection of any specific receptor for formyl peptide present in myeloblasts. Under these conditions, a broad band with average mol wt of 80 kD (Fig 2, lane A), which was only slightly decreased by a 1,000-fold excess of F-Met Leu Phe (Fig 2, lane B), was labeled in day 0 myeloid precursors. In contrast, the intense labeling of a band at 55 to 70 kD seen in day 8 cultures (Fig 2, lane C) was completely inhibited by an excess of F-Met Leu Phe (Fig 2, lane D). This band is identical in mol wt to specific receptor for formyl peptide seen in maturing myeloid cells and peripheral blood granulocytes shown in Fig 1.

DISCUSSION

In this report, we have demonstrated that formyl peptide receptors, which are present in large numbers on the surface of the segmented neutrophil,[1] are not detectable in normal myeloblasts and gradually increase in number as the cells mature. This observation fits well with our recent finding that while the binding of F-Met Leu Phe to the surface of the mature neutrophil is followed within seconds by membrane depolarization and a transient elevation in $[Ca^{2+}]_i$, these responses are totally absent in normal myeloblasts and evolve incrementally during cellular maturation.[6] Our ability[5] to carry out studies like these on the ontogenetic maturation of the granulocyte using normal human cells has only recently been developed and has resulted from the acquisition of monoclonal antibodies directed against differentiation antigens on most maturing hemic cells.

Before the development of this technique, information regarding the maturation of structure and function in granulocytes was inferred from studies using continuous malignant cell lines like the acute promyelocytic leukemic line HL-60, which can be induced to differentiate by a variety of polar solvents.[8] Using this approach, several groups demonstrated the gradual acquisition of receptor-mediated fluxes in $[Ca^{2+}]_i$,[9] membrane depolarization,[10,11] chemotaxis,[12] phagocytosis,[8] and superoxide anion (O_2^-) generation[13] as HL-60 cells are stimulated to mature in vitro along the granulocyte pathway.

In 1980, Niedel et al[14] showed that high affinity formyl peptide receptors were present on a subpopulation of undifferentiated HL-60 cells and that they increased 70- to 100-fold when the cells were caused to differentiate along the granulocyte pathway. The receptor-bearing cells, while varying in number from experiment to experiment, amounted to ≈10% of the total number of cells in unstimulated cultures and were said to resemble well differentiated promyelocytes. The number of receptors on the subpopulation of undifferentiated cells ranged from >300 to 15,000 per cell, while after the cells were induced to differentiate, the density increased to ≈200,000 per cell.

Our results confirm that the maturational development of formyl peptide receptor expression is similar in normal granulocytes to that predicted by the HL-60[15] model and provide further insight into the level at which the receptors may first appear. Unlike unstimulated HL-60 cells, the cell fraction that we harvest contains practically no cells with granules, since very few are more mature than myeloblasts. However, after 24 hours of exposure to G/M-CSFs, promyelocytes become the predominant type of cell present. By the technique of covalent affinity labeling that we used, specific formyl peptide receptors were undetectable on day 0 but clearly discernible 24 hours later. While specific peptide receptors may be present at the level of the myeloblast in amounts below the resolution of our assay, our results support the notion that the appearance of substantial numbers of receptors during the granulocyte's ontogeny awaits the acquisition of granules by the cell.

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CORRESPONDENCE

VP-16 IN BONE MARROW PURGING

To the Editor:

We would like to advise caution in the over-interpretation of the data of Kushner et al,[1] regarding the use of long-term marrow cultures (LTMC) to test the efficacy of VP16-213 (VP16) as an ex vivo bone marrow (BM) purging agent. Our data has established the efficacy of VP16 as an effective purging agent both in an in vivo murine model in which up to 50% of contaminating leukemia cells were effectively purged ex vivo from syngeneic BM,[2,3] and in vitro against several human leukemia and lymphoma cell lines.[2] However, unlike the activated cyclophosphamide derivatives,[4] we have found no differential kill of committed (CFU-GM) *v* pluripotent (CFU-S) stem cells at purging concentrations of VP16 >10 μg/mL.[2] As anticipated, the engraftment of VP16-treated BM in the mouse correlated directly with CFU-GM/CFU-S numbers transplanted.[3] Kushner et al demonstrated viable CFU-GM in LTMC for BM treated at VP16 concentrations that killed more than two logs of CFU-GM after a one-hour incubation. Yet, in our murine model, this degree of CFU-GM kill correlated directly with CFU-S kill and did not lead to successful engraftment, even when mice were transplanted with 16.7×10^8 cell/kg While LTMC data may be useful when evaluating activated cyclophosphamide derivatives as BM purging agents, we would advise extreme caution in treating patients using VP16-treated BM at concentrations that kill more than one log of CFU-GM

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To the Editor:

We emphasize in our report the same concerns as Dr Stiff Caution must be the rule in applying laboratory findings to clinical purging protocols. We recognize Dr Stiff's early work using VP-16 as a purging agent in mouse models. Stem cell assays in animals, however, cannot be completely relied on to correlate with data obtained in human marrow studies. Moreover, variability of in vitro data generated in different laboratories is the expected consequence of differences in biological reagents and culture conditions. For example, in our preclinical studies, we found that 4-hydroperoxycyclophosphamide (4-HC),[1] was markedly more toxic to CFU-GM than was reported by the Johns Hopkins group, and we therefore purge with a lower dose of 4-HC. Nevertheless, our observations that long-term marrow cultures may be more sensitive than clonal assays for assessing human hematopoietic regenerative capacity have been confirmed in separate studies by Ciobanu et al.[2] We are currently testing clinically the validity of our in vitro observations. In phase I trials for purging lymphocytic leukemic or lymphoma cells from harvested human marrow, we incubate 20×10^6 marrow cells per milliliter for one hour with a fixed dose of 100 μmol 4HC, plus VP-16 at doses that are increased by small increments after engraftment has been assured at one dose level in a sufficient number of patients The current level is 8.5 μmol VP-16, and no failures of hematopoietic reconstitution have occurred.

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MODELS OF PLATELET FORMATION

To the Editor:

In a recent in vitro study of megakaryocyte morphogenesis stimulated by thrombocytopenic plasma, Drs Leven and Kee proposed a model of platelet formation which they claim is consistent with available data.[1] This statement arises from two major preconceptions. The first of these is that platelets are formed in the bone marrow. The second is that cultured megakaryocytes mimic their in vivo counterparts

There is evidence that megakaryocytes are capable of squeezing through the blood/bone marrow barrier as complete cells without fragmentation.[2] Megakaryocyte cytoplasm can stream away from its nucleus and leave it behind in the marrow.[3] Entire megakaryocytes have been found in the sinusoidal spaces[3] and are carried away into the central venous blood.[4] The majority of megakaryocytes in central venous blood possess copious cytoplasm.[5] This is lost in the pulmonary microcirculation since mainly naked nuclei are found in arterial blood.[5] Megakaryocytes and large cytoplasmic fragments are found in the pulmonary circulation[3,6,7] and their number depends on the central venous blood supply.[8] The platelet volume distributions of rat, rabbit, and humans can be generated from megakaryocyte cytoplasmic volumes by a sequence of random binary divisions[9,10] at the bifurcations in the pulmonary microcirculation. This simple method of platelet formation provides a physical analogue of mitosis, explains the need for circulating megakaryocytes, and allows the maintenance of normal platelet counts without bone marrow platelet production.[9] As Drs Leven and Kee suggest,[1] it is possible that certain factors in plasma, which are magnified in thrombocytopenic states, do change the megakaryocyte cytoplasm between a "gel-like" and "sol-like" state.[11] With these changes the process of random sequential binary division of megakaryocyte cytoplasm provides an explanation for the platelet volume distributions observed in acute thrombocytopenia.[12]

It is important that different mechanisms of platelet production should be critically examined, but in doing so, all not just some, of the available data should be considered.

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To the Editor:

There are several ideas as to how platelets form. The four major models are by pseudopodia formation,[1-3] pinching off of cytoplasmic blebs from the megakaryocyte surface,[4-6] rapid dissolution of the megakaryocyte into numerous small fragments,[7-9] and pulmonary platelet formation.[10,11] Our recent report[3] describes the first of these four mechanisms. Consequently, we considered available data pertinent only to this one model, not all possible models of platelet formation. There is ample evidence in the literature showing that bone marrow megakaryocytes form elongate, beaded cytoplasmic extensions both in vivo and in vitro. We have shown[3] that fragments as small as two linked platelets can detach from pseudopodia with intact microtubule rings. In addition, the pseudopodia and fragments are similar to platelets in both ultrastructural appearance and response to platelet activators (unpublished observations). Our model for platelet formation does not necessarily imply that fragments the size of individual platelets form within the marrow. Indeed, long strings of attached platelets could be released that fragment into individual platelets in the circulation The pulmonary capillaries are one possible location, although the work of Tong et al[12] shows that large proplatelets may pass through the pulmonary circulation without fragmentation. Since small strings, three to five platelets in length, are commonly seen in our cultures, the majority of the fragmentation process can occur with no physical interaction of megakaryocytes with other tissues. The location and mechanism of fragmentation into individual platelets in vivo cannot be answered by our studies and remains to be determined.

Megakaryocyte fragmentation in the pulmonary circulation is another possible mechanism for platelet formation. Dr Trowbridge has described the data that support this model, but there are certain problems that should be pointed out as well. One is that no ultrastructure study has documented megakaryocyte fragmentation in the lung. Also, it has been assumed that naked megakaryocyte nuclei in the lung are the result of platelet shedding at this location, yet one must consider that they may arrive in the lung as naked nuclei from the bone marrow. The major evidence supporting pulmonary platelet formation has been the difference in platelet count across the pulmonary vascular bed. Yet this difference may be due to sampling artefact or bias as stated by Martin et al.[13] Finally, if one calculates platelet production based on Tinggaard Pederson's value of 11.9 megakaryocytes/mL[14] one cannot account for total platelet production from circulating megakaryocytes.

Data both in favor of and contrary to all models of platelet formation are available, but as yet, there is no evidence to indicate that platelets form by only one mechanism. We believe it is most prudent, until evidence to the contrary exists, to assume that there may be more than one mechanism by which platelets are formed.

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EXPRESSION OF IMMUNOLOGIC MARKERS IN MEGAKARYOBLASTS

To the Editor·

We read with interest the recent paper by Koike et al[1] in which they analyzed the expression of myeloid markers on megakaryoblasts and proposed a model for the order of appearance of markers in megakaryoblast maturation. We would like to comment on the immunophenotype of megakaryoblasts based on our experience with 27 of 240 patients with acute leukemia other than lymphoblastic in which a megakaryoblastic component was detected All cases were studied systematically with a panel of 22 monoclonal antibodies in which included four specific for platelet glycoproteins (GP). two against GP IIb/IIIa (CDw41), one for GP Ib (CDw42), and one for GP IIIa.

Regarding the expression of myeloid markers on megakaryoblasts, from our study, and according to Koike et al,[1] it can be concluded that megakaryoblasts are generally negative for the CD13 antigen, and this antigen would thus be restricted to the granulomonocytic lineage. Nevertheless, in two of the 27 cases there was a clear-cut overlap of MY7 (CD13) and J15 (CDw41) positive cells, which suggests that individual blast cells simultaneously express granulomonocytic and platelet antigens, although specific double-labeling was not performed. In addition, our results show that megakaryoblasts are also negative for specific monocytic (CD14) and granulocytic (CD15) markers

Conflicting results have been reported regarding HLA-DR expression by megakaryoblasts,[2-4] and the possibility of its being the first antigen expressed in this lineage, which would be lost subsequently, as proposed by Koike et al,[1] is attractive. Nevertheless, the results from one of our patients suggest that on some occasions at least, the expression of GP IIb/IIIa may precede that of HLA-DR In our patient, J15 (GP IIb/IIa) detected twice the number of cells, as compared with FMC25, which would point to a relatively immature population of megakaryoblasts[3]; these cells were almost completely negative for HLA-DR In Koike's model the expression of GP IIIa, probably the first GP to be acquired by the megakaryoblast,[4] is not considered In one of our pure megakaryoblastic cases, the cells displayed a high reactivity for GP IIIa (70% of the blast cells) while GP IIb/IIIa and GP Ib were expressed in a minority of cells (15%), indicating that we were dealing with very early megakaryoblasts[4] These cells were lacking in HLA-DR and CD34 (BI-3C5) antigens, which would indicate that GP IIIa is acquired earlier than the HLA-DR marker in the megakaryoblasts maturation.

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To the Editor:

We appreciate the comments of San Miguel et al regarding our recent publication in *Blood*.[1] They pointed out two possibilities: (1) expression of CD13 on megakaryoblasts, and (2) earlier appearance of glycoprotein(GP)IIIa than HLA-DR on immature cells of megakaryocytic lineage.

Their finding that CD13 was expressed on blasts positive for GPIIb/IIIa in some patients with megakaryoblastic leukemia is very interesting. Such cases may be rare, as they indicated (about 7%), and may be due to aberrant expression, as observed with CD13 on common ALL cells.[2] We reported that CD33 antigen was expressed on some megakaryoblasts which were identified by both platelet peroxidase (PPO) activity and anti-GPIIb/IIIa reactivity.[1] Breton-Gorius et al[3] also recently described the presence of CD33 on megakaryoblasts. It is well known that CD33 is expressed not only on cells of the myeloid series, including CFU-GM, but also on some BFU-E or some CFU-GEMM. Therefore, it is reasonable to speculate that at least some megakaryocytic precursors have CD33. On the other hand, CD13 is thought to be expressed only on the myeloid series in normal hematopoietic cells, and not on CFU-GEMM. Whether CD13 is expressed on normal megakaryocytic precursors should be confirmed by megakaryocyte colony-forming assay using normal bone marrow cells treated with monoclonal antibodies and complement. I would like to stress that MPO-negative, CD13 or CD14-negative, and CD33-positive blasts should be tested for PPO reaction and anti-GPIIb/IIIa antibodies.

For the identification of megakaryoblasts, the PPO reaction and anti-GP antibodies, especially anti-GPIIb/IIIa, are useful.[1,3-6] If many blasts are positive for both PPO and GPIIb/IIIa, the diagnosis is megakaryoblastic leukemia. In such cases, the proportion of blasts positive for HLA-DR antigen is relatively small compared with that of blasts positive for GPIIb/IIIa in our experience.[1,4,5] On the other hand, some cases lack GPIIb/IIIa or GPIIIa but express PPO.[1,3-5] Such cases would be left undetected when cell surface phenotyping is only used for diagnosis of megakaryoblastic leukemia. Breton-Gorius et al reported that such cases account for ~20% of all cases of acute megakaryoblastic leukemia.[3,6] It is noteworthy that HLA-DR antigens are often positive on these blasts.[1,3,4] HLA-DR antigens are thought to be expressed on CFU-GEMM. Therefore, PPO-positive, HLA-DR-positive, GPIIb/IIIa-negative blasts may be less mature megakaryoblasts than blasts positive for GPIIb/IIIa. Although we have not used anti-GPIIIa antibodies for the identification of megakaryoblasts, we speculate that the megakaryoblasts positive for GPIIIa but negative for GPIIb/IIIa or HLA-DR are slightly less mature than those positive for GPIIb/IIIa, but more mature than those positive only for PPO and HLA-DR.

It should be noted that PPO-like activity was recently detected in erythroid precursors.[7,8] It is necessary to rule out the early phase of erythroleukemia (cryptic erythroleukemia) when blasts lack GPIIb/IIIa but express PPO. We tested the reactivity of blasts for 5F1[9] (donated by Dr Andrews) and EP1[10] (donated by Dr Yokochi) by the APAAP method using cryopreserved smears from four patients in which most of blasts were positive for PPO and HLA-DR but negative for GPIIb/IIIa. In these patients, blasts were negative for 5F1 and EP1, and they lacked theta granules containing ferritin,[8] a characteristic feature of early erythroblasts. It was reported that erythroblasts with PPO-like activity were usually devoid of HLA-DR.[8,11] Therefore, it is unlikely that these cases were erythroleukemia.

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ANNOUNCEMENTS

AMERICAN SOCIETY OF HEMATOLOGY
TWENTY-NINTH ANNUAL MEETING
Washington, DC
December 5-8, 1987
SUMMARY OF PROGRAM

Saturday, December 5
Morning: EDUCATION PROGRAM
- Hemoglobins
- Acute Leukemia
- Molecular Genetics
- Immune Markers in the Diagnosis and Treatment of Hematopoietic Malignancies
- Thrombosis
- Platelets
- Transfusion Medicine
- Hodgkin's Disease
- Oncogenes in Hematopoietic Neoplasms
- Biological Response Modifiers
- Laboratory Medicine
- Marine Lipids

Afternoon: EDUCATION PROGRAM
- Pediatric Hematology
- Bone Marrow Transplantation
- Retroviruses in AIDS and Hematologic Malignancy
- Immune Markers in the Diagnosis and Treatment of Hematopoietic Malignancies
- Bleeding Disorders
- Ham Wasserman Lecture: Glucose 6-Phosphate Dehydrogenase (G6PD) Deficiency: Clinical Expression of Multiple Genetic Defects
- Hodgkin's Disease
- Laboratory Medicine
- Marine Lipids

SCIENTIFIC SUBCOMMITTEE SYMPOSIA
- Subcommittee on Neoplasia
- Subcommittee on Pediatric Hematology
- Subcommittee on Platelets
- Subcommittee on Hemostasis
- Subcommittee on Transfusion Medicine
- Council on Thrombosis "How To" Session

FORUM ON THE PRACTICE OF HEMATOLOGY

PM: Council on Thrombosis
- Advances in Chemotherapy for Hodgkin's and Non-Hodgkin's Lymphomas sponsored by Bristol Meyers

Evening: Opening Reception

Sunday, December 6
Morning: EDUCATION PROGRAM
- Transfusion Medicine
- Bone Marrow Transplantation
- Biological Response Modifiers
- Molecular Genetics
- Pediatric Hematology
- Platelets
- Hemoglobins
- Acute Leukemia
- Retroviruses in AIDS and Hematologic Malignancy
- Oncogenes in Hematopoietic Neoplasms
- Bleeding Disorders
- Thrombosis
- NIH Grants Workshop

SCIENTIFIC SUBCOMMITTEE SYMPOSIA
- Subcommittee on Hemoglobin/Red Cell
- Subcommittee on Hematopoietic Growth Factors
- Subcommittee on Thrombosis
- Subcommittee on Immunohematology

Afternoon:
- **FIRST BUSINESS MEETING**
- **PRESIDENT'S REPORT TO THE SOCIETY**
- Simultaneous Scientific Sessions
- Scientific Poster Sessions

Monday, December 7
Morning
- Simultaneous Scientific Sessions
- Scientific Poster Sessions

Afternoon
- Simultaneous Scientific Sessions
- Scientific Poster Sessions

Evening
- Guarneri String Quarter Concert

Tuesday, December 9
Morning
- Stratton Lecture
- Dameshek Award
- Presidential Symposium
- General Business Meeting
- Scientific Poster Sessions

Afternoon
- 4 Mini-Plenary Sessions

WILMOT CANCER RESEARCH FELLOWSHIPS

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For further information, contact: Wilmot Cancer Research Fellowship Program, Medical Center Box 601, University of Rochester School of Medicine & Dentistry, 601 Elmwood Ave, Rochester, NY 14642.

ONCOLOGY PRACTICE 1988: A PERSPECTIVE

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For further information, contact· Stephen E. Jones, MD, Charles A. Sammons Cancer Center, Room 4800, Baylor University Medical Center, 3500 Gaston Ave, Dallas, TX 75246 Phone: (214) 820-2021.

APLASTIC ANEMIA FOUNDATION OF AMERICA (AAFA) RESEARCH FELLOWSHIP AWARD

The AAFA has established a Research Fellowship Award in order to encourage young researchers and to recognize their contributions to research related to aplastic anemia. In January 1988, an award of $25,000 will be made for a research fellowship to begin July 1988. The deadline for completion of the application package is November 15, 1987.

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HOUSING ARRANGEMENTS FOR THE 1987 ANNUAL MEETING OF THE AMERICAN SOCIETY OF HEMATOLOGY

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For further information, contact: Mr. Claude M. Weil, Tutorial Administrator, c/o International House of Chicago, 1414 East 59th Street, Chicago, IL 60637, USA. Telephone (312) 753-2277.

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BLOOD	Monthly
COMPREHENSIVE PSYCHIATRY	Bimonthly
INFORMATICS IN PATHOLOGY	Quarterly
JOURNAL OF CARDIOTHORACIC ANESTHESIA	Bimonthly
JOURNAL OF CLINICAL ONCOLOGY	Monthly
JOURNAL OF CRITICAL CARE	Quarterly
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JOURNAL OF PEDIATRIC SURGERY	Monthly
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SEMINARS IN ARTHRITIS AND RHEUMATISM	Quarterly
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SEMINARS IN DIAGNOSTIC PATHOLOGY	Quarterly
SEMINARS IN HEMATOLOGY	Quarterly
SEMINARS IN NEPHROLOGY	Quarterly
SEMINARS IN NUCLEAR MEDICINE	Quarterly
SEMINARS IN ONCOLOGY	Quarterly
SEMINARS IN ONCOLOGY NURSING	Quarterly
SEMINARS IN OPHTHALMOLOGY	Quarterly
SEMINARS IN ORTHOPAEDICS	Quarterly
SEMINARS IN PERINATOLOGY	Quarterly
SEMINARS IN RESPIRATORY INFECTIONS	Quarterly
SEMINARS IN ROENTGENOLOGY	Quarterly
SEMINARS IN ULTRASOUND, CT, AND MR	Quarterly
SEMINARS IN UROLOGY	Quarterly
SEMINARS IN VETERINARY MEDICINE AND SURGERY (SMALL ANIMAL)	Quarterly
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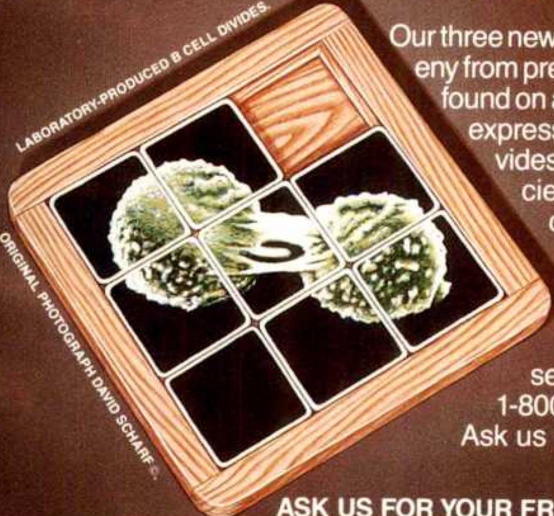

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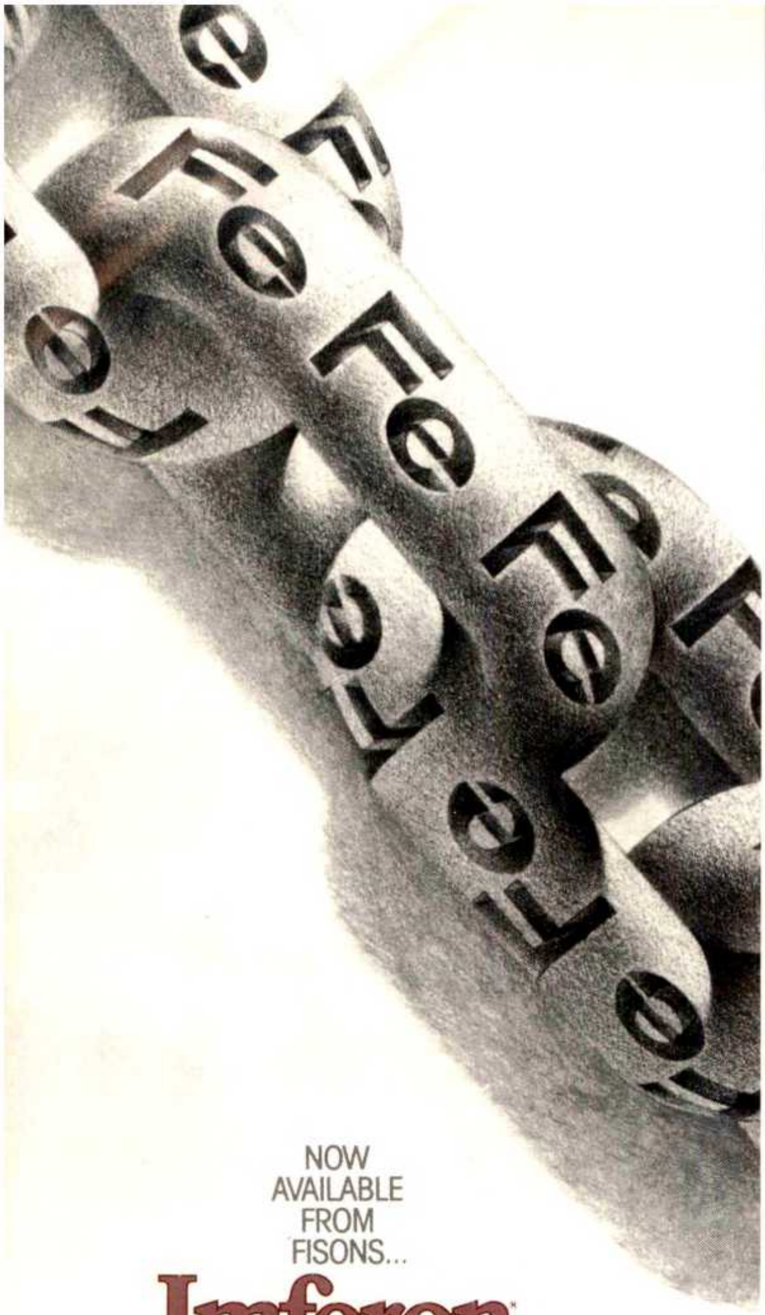

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William G. Hocking, M.D.
Department of Clinical Oncology
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1000 North Oak Avenue
Marshfield, WI 54449

MarshfieldClinic

Read this like your life depends on it.

Breast cancer found early and treated promptly has an excellent chance for cure. About a week after your period practice this self-examination.

1. In bath or shower.
Fingers flat, move opposite hand gently over each breast. Check for lumps, hard knots, thickening.

2. In front of a mirror.
Observe breasts. Arms at sides. Raise arms high overhead. Any change in nipples, contours, swelling, dimpling of skin? Palms on hips: press down firmly to flex chest muscles.

3. Lying down.
Pillow under right shoulder, right hand behind head. Left hand fingers flat, press gently in small circular motions starting at 12 o'clock. Make about three circles moving closer to and including nipple. Repeat on left.

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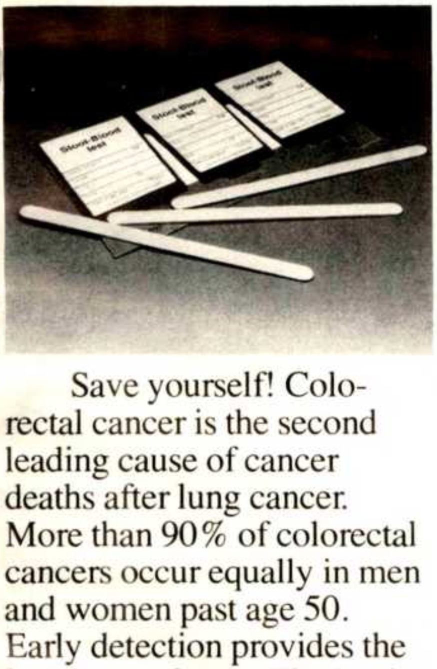
50,000 people will be saved from colorectal cancer this year. You can save one.



Save yourself! Colorectal cancer is the second leading cause of cancer deaths after lung cancer. More than 90% of colorectal cancers occur equally in men and women past age 50. Early detection provides the best hope of cure. That's why if you're over 50, you should take this simple, easy Stool Blood Test every year. The test kit is chemically treated to detect hidden blood in the stool and can be done at the time of your periodic health examination so your doctor will know the results.

Two days before the test, you begin a diet you might enjoy all the time. Plenty of fresh vegetables raw or cooked, especially corn, spinach and lettuce. Lots of plums, grapes, apples and prunes, moderate amounts of peanuts and popcorn. No red meat, turnips or horseradish. Do's and don't's are listed in the kit.

The presence of hidden blood usually indicates some problem in the stomach or bowel, not necessarily cancer. Positive tests must be followed by further testing to find out what the problem is.

Other tests for colorectal cancer you should talk to your doctor about: Digital rectal exam (after age 40); the procto test (after age 50). It is important to report any personal or family history of intestinal polyps or ulcerative colitis, and any change in your bowel habits, which could be a cancer warning signal.

The American Cancer Society wants you to know.

Kamen Norman Ng

AMERICAN CANCER SOCIETY®

This space contributed as a public service.

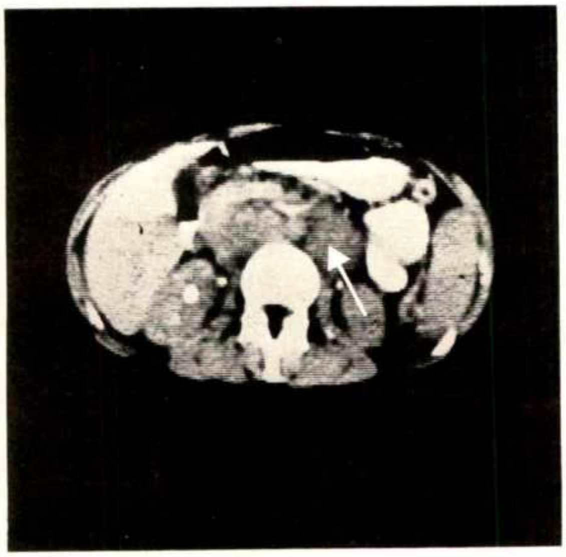
Blenoxane® *Combinations*

(sterile bleomycin sulfate)

Proven Effective in Non-Hodgkin's Lymphoma

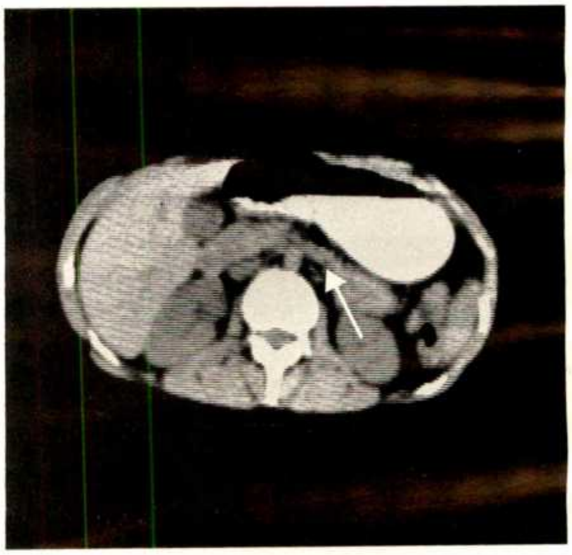
The newer Blenoxane containing combinations resulted in high complete response rates and extended disease-free survival.[1-7]

Abdominal/Pelvic CT
February 25



Massive retroperitoneal, retrocrural, para-aortic, and probable right iliac adenopathy.

Abdominal/Pelvic CT
August 4



No evidence of abnormal abdominal or pelvic adenopathy after Blenoxane combination chemotherapy.

CHOP-Bleo

1. Newcomer LN, Cadman EC, Nerenberg MI, *et al:* Randomized study comparing doxorubicin, cyclophosphamide, vincristine, methotrexate with leucovorin rescue, and cytarabine (ACOMLA) with cyclophosphamide, doxorubicin, vincristine, prednisone, and bleomycin (CHOP-B) in the treatment of diffuse histiocytic lymphoma. *Cancer Treat Rep* 66:1279-1284, 1982.

BACOP

2. Schein PS, DeVita VT Jr, Hubbard S, *et al:* Bleomycin, Adriamycin, cyclophosphamide, vincristine, and prednisone (BACOP) combination chemotherapy in the treatment of advanced diffuse histiocytic lymphoma. *Ann Intern Med* 85:417-422, 1976.

COP-BLAM

3. Laurence J, Coleman M, Allen SL, *et al:* Combination chemotherapy of advanced diffuse histiocytic lymphoma with the six-drug COP-BLAM regimen. *Ann Intern Med* 97:190-195, 1982.

*Case information on file at Bristol-Myers Oncology Division

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Blenoxane® ...Integral Component of Chemotherapy Regimens in Non-Hodgkin's Lymphoma

(sterile bleomycin sulfate)

Since Blenoxane is virtually non-myelosuppressive, continuing therapy with certain Blenoxane containing chemotherapy regimens (e.g. BACOP) can prevent tumor regrowth between cycles of other chemotherapy regimens while allowing for bone marrow recovery.[*2,3,4,6]

Blenoxane can produce, with cumulative doses, a syndrome of pulmonary fibrosis. It is usually dose-related, being seen infrequently in patients whose total dose of Blenoxane is less than 400 units.

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M-BACOD

4. Skarin AT, Canellos GP, Rosenthal DS, *et al:* Improved prognosis of diffuse histiocytic and undifferentiated lymphoma by use of high dose methotrexate alternating with standard agents (M-BACOD). *J Clin Oncol* 1:91-98, 1983.
5. Anderson KC, Skarin AT, Rosenthal DS, *et al:* Combination chemotherapy for advanced non-Hodgkin's lymphomas other than diffuse histiocytic or undifferentiated histologies. *Cancer Treat Rep* 68:1343-1350, 1984.

m-BACOD

6. Skarin A, Canellos G, Rosenthal D, *et al:* Moderate dose methotrexate (m) combined with bleomycin (B), Adriamycin (A), cyclophosphamide (C), Oncovin (O) and dexamethasone (D), *m*-BACOD, in advanced diffuse histiocytic lymphoma (DHL). *Proc Am Soc Clin Oncol* 2:220, 1983.

MACOP-B

7. Klimo P, Connors JM: MACOP-B chemotherapy for the treatment of diffuse large-cell lymphoma. *Ann Intern Med* 102:596-602, 1985.

See prescribing information on following page.

Blenoxane® *(sterile bleomycin sulfate)*

Brief Summary of Prescribing Information (1) 10/84. For complete prescribing information please consult product literature.

WARNING

It is recommended that Blenoxane® be administered under the supervision of a qualified physician experienced in the use of cancer chemotherapeutic agents. Appropriate management of therapy and complications is possible only when adequate diagnostic and treatment facilities are readily available.

Pulmonary fibrosis is the most severe toxicity associated with Blenoxane. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Its occurrence is higher in elderly patients and in those receiving greater than 400 units total dose, but pulmonary toxicity has been observed in young patients and those treated with low doses.

A severe idiosyncratic reaction consisting of hypotension, mental confusion, fever, chills, and wheezing has been reported in approximately 1% of lymphoma patients treated with Blenoxane.

INDICATIONS: Blenoxane should be considered a palliative treatment. It has been shown to be useful in the management of the following neoplasms either as a single agent or in proven combinations with other approved chemotherapeutic agents:

Squamous Cell Carcinoma — Head and neck including mouth, tongue, tonsil, nasopharynx, oropharynx, sinus, palate, lip, buccal mucosa, gingiva, epiglottis, skin, larynx, penis, cervix, and vulva. The response to Blenoxane is poorer in patients with head and neck cancer previously irradiated.

Lymphomas — Hodgkin's, reticulum cell sarcoma, lymphosarcoma.

Testicular Carcinoma — Embryonal cell, choriocarcinoma, and teratocarcinoma.

CONTRAINDICATIONS: Blenoxane is contraindicated in patients who have demonstrated a hypersensitive or an idiosyncratic reaction to it.

WARNINGS: Patients receiving Blenoxane must be observed carefully and frequently during and after therapy. It should be used with extreme caution in patients with significant impairment of renal function or compromised pulmonary function.

Pulmonary toxicities occur in 10% of treated patients. In approximately 1%, the nonspecific pneumonitis induced by Blenoxane progresses to pulmonary fibrosis, and death. Although this is age and dose related, the toxicity is unpredictable. Frequent roentgenograms are recommended.

Idiosyncratic reactions similar to anaphylaxis have been reported in 1% of lymphoma patients treated with Blenoxane. Since these usually occur after the first or second dose, careful monitoring is essential after these doses.

Renal or hepatic toxicity, beginning as a deterioration in renal or liver function tests, have been reported, infrequently. These toxicities may occur, however, at any time after initiation of therapy.

Usage in Pregnancy: Safe use of Blenoxane in pregnant women has not been established.

ADVERSE REACTIONS: Pulmonary — This is potentially the most serious side effect, occurring in approximately 10% of treated patients. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Approximately 1% of patients treated have died of pulmonary fibrosis. Pulmonary toxicity is both dose and age-related, being more common in patients over 70 years of age and in those receiving over 400 units total dose. This toxicity, however, is unpredictable and has been seen occasionally in young patients receiving low doses.

Because of lack of specificity of the clinical syndrome, the identification of patients with pulmonary toxicity due to Blenoxane has been extremely difficult. The earliest symptom associated with Blenoxane pulmonary toxicity is dyspnea. The earliest sign is fine rales.

Radiographically, Blenoxane-induced pneumonitis produces nonspecific patchy opacities, usually of lower lung fields. The most common changes in pulmonary function tests are a decrease in total lung volume and a decrease in vital capacity. However, these changes are not predictive of the development of pulmonary fibrosis.

The microscopic tissue changes due to Blenoxane toxicity include bronchiolar squamous metaplasia, reactive macrophages, atypical alveolar epithelial cells, fibrinous edema, and interstitial fibrosis. The acute stage may involve capillary changes and subsequent fibrinous exudation into alveoli producing a change similar to hyaline membrane formation and progressing to a diffuse interstitial fibrosis resembling the Hamman-Rich syndrome. These microscopic findings are nonspecific, e.g., similar changes are seen in radiation pneumonitis, pneumocystic pneumonitis.

To monitor the onset of pulmonary toxicity, roentgenograms of the chest should be taken every 1 to 2 weeks. If pulmonary changes are noted, treatment should be discontinued until it can be determined if they are drug related. Recent studies have suggested that sequential measurement of the pulmonary diffusion capacity for carbon monoxide (DL_{co}) during treatment with Blenoxane may be an indicator of subclinical pulmonary toxicity. It is recommended that the DL_{co} be monitored monthly if it is to be employed to detect pulmonary toxicities, and thus the drug should be discontinued when the DL_{co} falls below 30 to 35% of the pretreatment value.

Because of bleomycin's sensitization of lung tissue, patients who have received bleomycin are at greater risk of developing pulmonary toxicity when oxygen is administered at surgery. While long exposure to very high oxygen concentrations is a known cause of lung damage, after bleomycin administration, lung damage can occur at lower concentrations than usually would be considered safe. Suggested preventive measures are:

(1) Maintain FI O_2 at concentrations approximately that of room air (25%) during surgery and the postoperative period.

(2) Monitor carefully fluid replacement, focusing more on colloid administration rather than crystalloid.

Idiosyncratic Reactions — In approximately 1% of the lymphoma patients treated with Blenoxane an idiosyncratic reaction, similar to anaphylaxis clinically, has been reported. The reaction may be immediate or delayed for several hours, and usually occurs after the first or second dose. It consists of hypotension, mental confusion, fever, chills, and wheezing. Treatment is symptomatic including volume expansion, pressor agents, antihistamines, and corticosteroids.

Integument and Mucous Membranes — These are the most frequent side effects, being reported in approximately 50% of treated patients. These consist of erythema, rash, striae, vesiculation, hyperpigmentation, and tenderness of the skin. Hyperkeratosis, nail changes, alopecia, pruritus, and stomatitis have also been reported. It was necessary to discontinue Blenoxane therapy in 2% of treated patients because of these toxicities.

Skin toxicity is a relatively late manifestation usually developing in the 2nd and 3rd week of treatment after 150 to 200 units of Blenoxane have been administered and appears to be related to the cumulative dose.

Other — Fever, chills, and vomiting were frequently reported side effects. Anorexia and weight loss are common and may persist long after termination of the medication. Pain at tumor site, phlebitis, and other local reactions were reported infrequently.

There are isolated reports of Raynaud's phenomenon occurring in patients with testicular carcinomas treated with a combination of Blenoxane and Velban®. It is currently unknown if the cause for the Raynaud's phenomenon in these cases is the disease, Blenoxane, Velban, or a combination of any or all of these.

SUPPLY: Each vial contains 15 units of Blenoxane as sterile bleomycin sulfate. NDC 0015-3010-20

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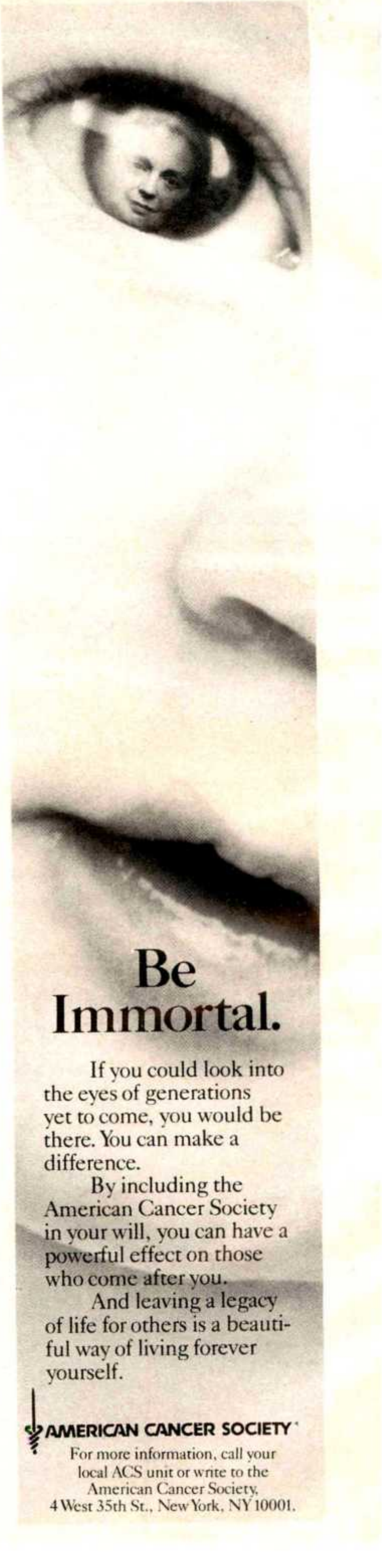
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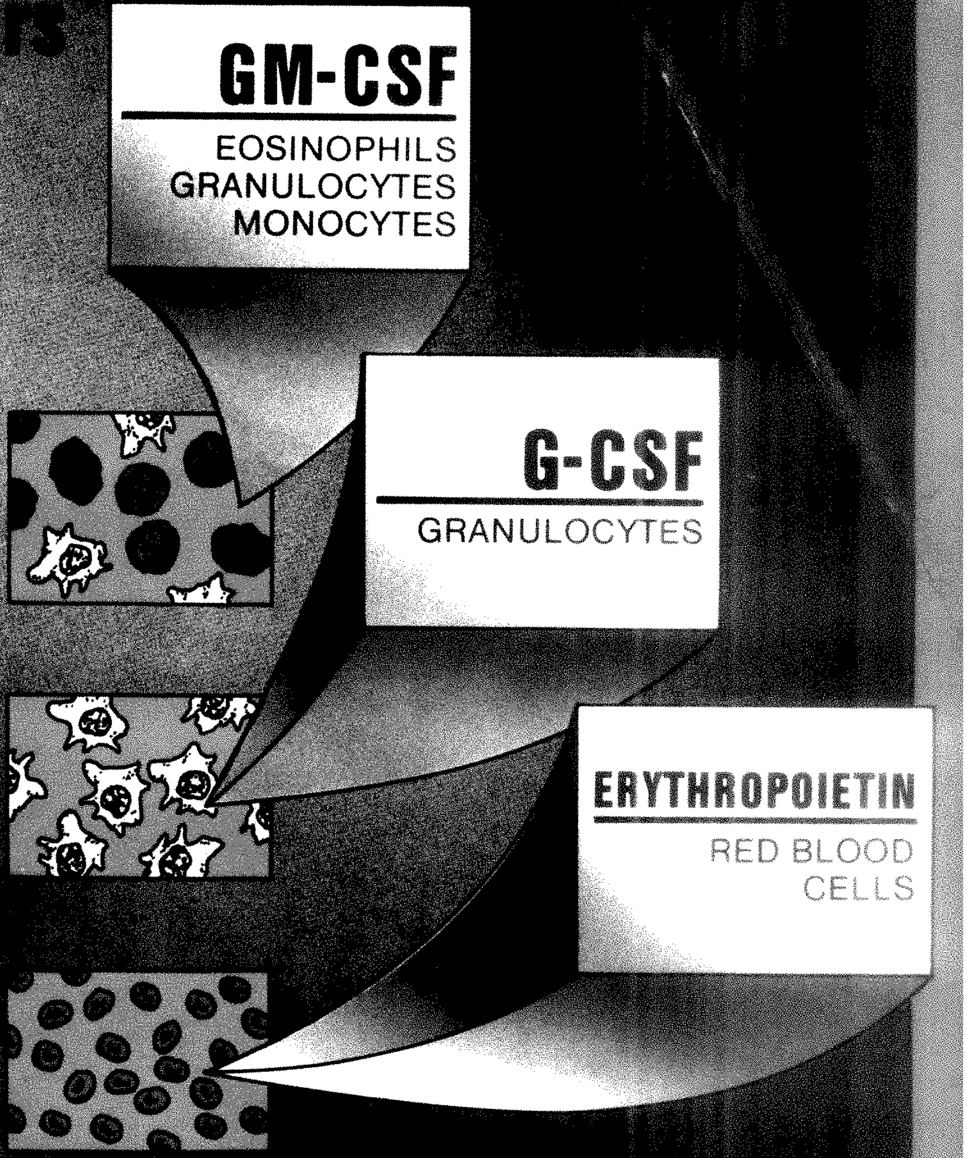

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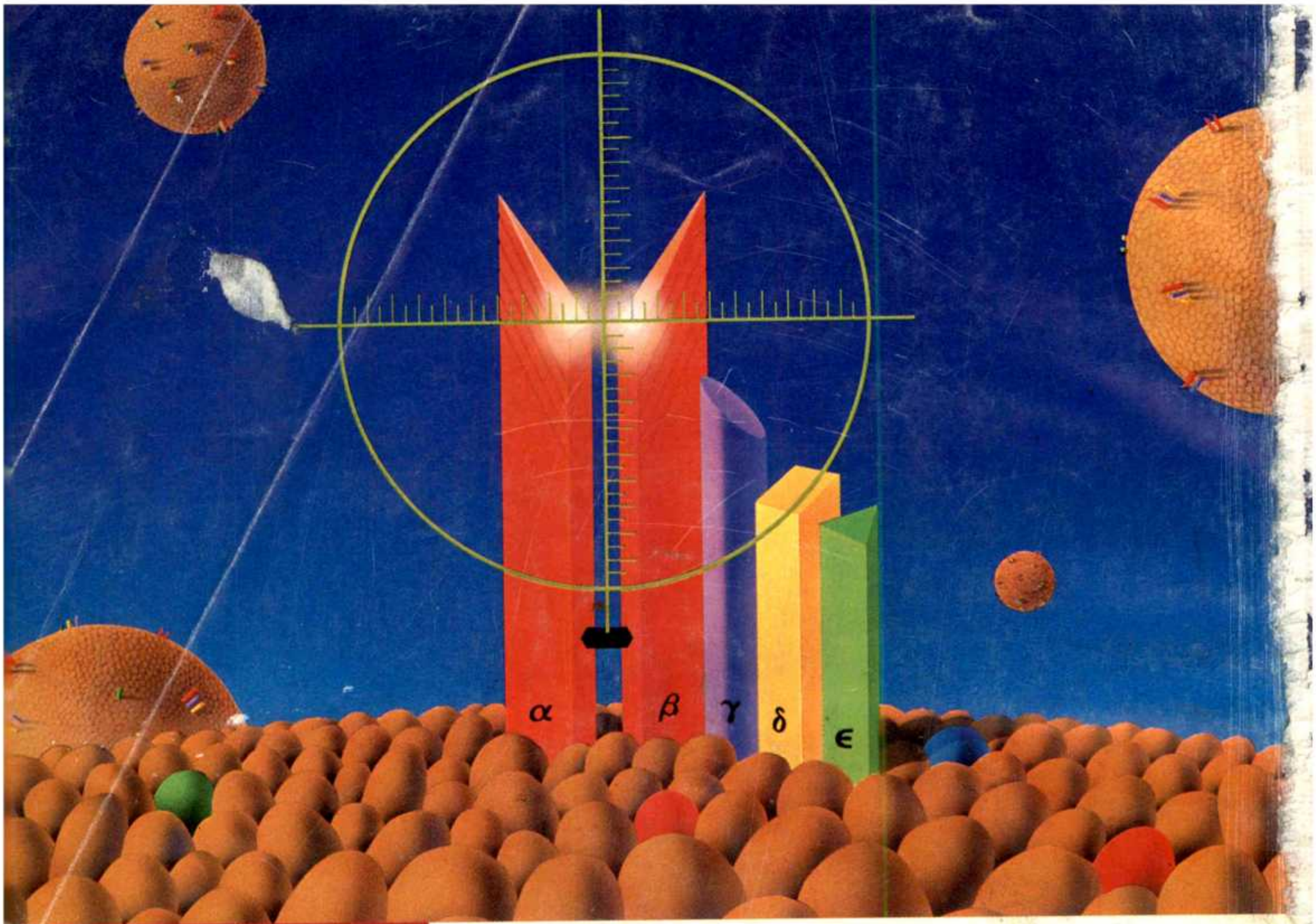

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